IXth ESMB meeting,
Nantes, 12-14 May 2002

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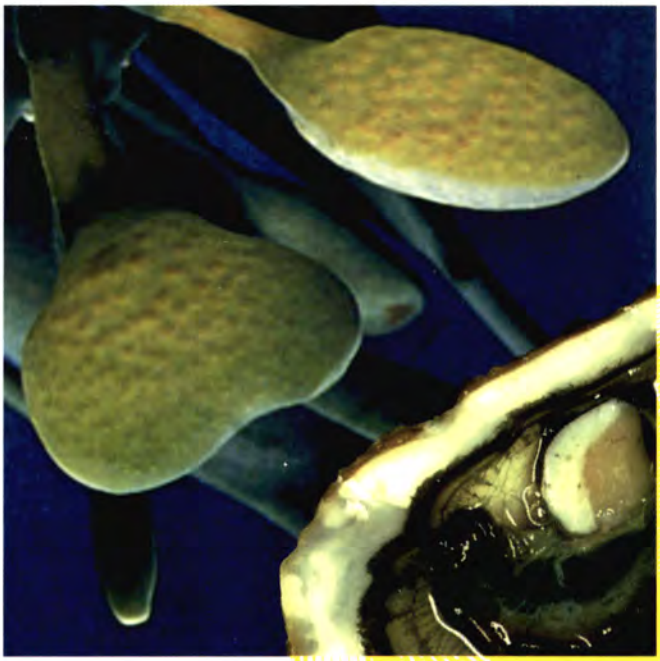

Marine biotechnology
an overview of leading fields

36

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Biotechnology

Ifremer

Marine biotechnology: an overview of leading field

IXth ESMB meeting
Nantes, 12-14 May 2002

Edited by
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With
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Ifremer

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Le comité d'organisation

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The Organizing Committee

Foreword

Marine environments represent unique ecosystems with a high diversity of organisms. These organisms include animals, plants and microorganisms from marine and esturian habitats along with extreme environments such as deep-sea hydrothermal vents from which special properties can be expected due to their living conditions.
For many years there is a growing interest in the discovery of metabolites of biotechnological interest from marine organisms. Marine organisms, from bacteria to eukaroytes, are undoubtedly a source of molecules of a great interest in biotechnology. Although numerous marine species synthesise biologically active molecules, continuation in looking for new bioactive substances from marine origin is promising, for example bioactive substances for pharmaceutical, medical, agricultural and environmental uses and for natural antifouling products. To date, about 30% of the drugs present on the market have been developed from natural products and so far less than 10% of the natural compounds have been isolated from marine organisms. In terms of marine microorganisms, less than 400 secondary metabolites have been isolated from marine bacteria and no more than 300 from marine fungi. Although in the past ten years there was an exponential increase in marine biotechnology, the search for the discovery of innovative metabolites from marine organisms can be considered only at the beginning. In that sea water can be considered as a very promising environment and new metabolites with biotechnological applications can be expected in the very near future.

Dr Jean Guézennec
Head of the Biotechnology and Marine Molecules Research Group

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Book:
Colliec-Jouault S., Bergé J.P., Guézennec J., Fleurence J., Le Gal Y., Roy P., 2003. Marine biotechnology: an overview of leading fields. Éd. Ifremer, Actes Colloq., 36, 186 p.

Paper:
Dumay, J., Bergé J.P., Debiton E., Barthomeuf C., 2003. SQDGs from *Porphyridium cruentum* inhibit the proliferation of human cancer cell-lines. *In:* Marine biotechnology : an overview of leading fields. Colliec-Jouault S., Bergé J.P., Guézennec J., Fleurence J., Le Gal Y., Roy P. (eds). Éd. Ifremer, Actes Colloq., 36, 17-23.

Chapter I

Marine organisms and bioactive compounds

Enzymes from marine microorganisms

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Introduction

For the environmental friendly and economic production of bulk and fine chemicals the use of biotechnological processes (in special biotransformations) becomes more and more important. Thus, there is an increasing demand of high specific and selective enzymes for the production of such components (e.g. chirale amines, alcohols, bromated or chlorinated amino acids). In industry especially the enzyme systems isolated from *Corynebacterium glutamicum* are in use for the set-up of new biotransformation. However, marine microorganisms offer a great source of new or more active enzymes. As an example the enzyme sulphite oxidase from *Sulfitobacter pontiacus* will be described in this paper.

Sulphite oxidase

Sulphite oxidase (sulphite acceptor oxidoreductase) catalyses the oxidation of sulphite to sulphate (Rajagopalan, 1980). The enzyme transfers electrons to oxygen, cytochrome c and a variety of other electron acceptors. In mammalian tissues the physiological importance of sulphite oxidase is due to its role as a terminal enzyme in the degradation of sulphur containing amino acids. Furthermore it is important in the detoxification of endogenous sulphite and sulphur dioxide (Parini *et al.*, 1997), a sulphite oxidase deficiency in the human organism leads to severe neurological disorders (Edwards *et al.*, 1999; Rupar *et al.*, 1996). Sulphite oxidase has been located in several mammalian tissues such as chicken liver (Kipke *et al.*, 1989), bovine liver (Fridovich & Cohen, 1971), rat liver (Rajagopalan & Johnson, 1977; Johnson & Rajagopalan, 1976; Southerland & Rajagopalan, 1978) and human liver (Johnson & Rajagopalan, 1976), but also in plants (Jolivet *et al.*, 1995) and in bacteria. Sugio *et al.* (1988) found a sulphite oxidase in *Thiobacillus ferrooxidans* and Kappler *et al.* (2000) isolated a sulphite oxidase from *Thiobacillus novellus*. Also many phototrophic bacteria contain sulphite oxidases (Brune, 1989, 1995). Sulphite oxidase is commercially available (sulphite oxidase from chicken liver, Sigma) and apart from preparations containing only the purified enzyme there are also preparations available where sulphite oxidase is the key component in an enzyme based analytical sulphite test kit (sulphite test kit, r-Biopharm). Due to its toxicity in higher concentrations (Baker *et al.*, 1981; Stevenson & Simon, 1981; Simon *et al.*, 1982) sulphite is an important analyte especially in the food and pharmaceutical industries. Since the Food and

Drug Administration (FDA) in the USA has established 10 ppm as the threshold for declaration of sulphite in foods, non-alcoholic beverages and wine products (Code of Federal Regulations, 2001) great efforts are invested in the construction of sulphite biosensors based on sulphite oxidase as the biological compound (Smith, 1987; Sasaki *et al.*, 1997; Situmorang *et al.*, 1999; Ng *et al.*, 2000). The products so far commercially available as well as the biosensors in development make use of chicken liver sulphite oxidase. In our work we focus on sulphite oxidase from the marine bacterium *Sulfitobacter pontiacus*.

Cultivation of *Sulfitobacter pontiacus*

Sulfitobacter pontiacus is a gram-negative bacterium that was isolated from water samples taken from a depth of 100-140 m at the H_2S-O_2 interface in the eastern part of the Black Sea (Sorokin, 1995). *Sulfitobacter pontiacus* is strict heterotrophic and is unable to grow autotrophically on H_2, thiosulphate or sulphite. The organism is strict aerobe and requires NaCl (5-80g/l, optimum 20-25g/l). Temperature and pH ranges are 4-35°C and pH 6.5-8.5. During metabolic studies of the organism Sorokin (1995) and Sorokin *et al.* (1999) found out that in acetate limited continuous culture *Sulfitobacter pontiacus* tolerates, after an adaptation period, extremely high sulphite concentrations of up to 63 mmol/l. Furthermore they found out that the oxidation of sulphite to sulphate by a highly active AMP-independent soluble sulphite oxidase leads to an increase in biomass concentration pointing to the ability of the organism to use sulphite as an additional source of energy. We performed studies on the growth conditions of *Sulfitobacter pontiacus* in batch culture in order to be able to produce and purify sulphite oxidase from this organism on a larger scale. We concentrated our investigations on the commercially available cultivation medium Marine Broth 2216 and focused on the influence of:

- the complexating agent EDTA;
- the concentration of the carbon source acetate;
- the concentration of the basic medium MB2216;
- sodium thiosulphate;
- HEPES as a buffering agent;
- the parameters temperature and oxygen supply on the performance of the cultivations in respect to biomass concentration and specific sulphite oxidase activity. In our initial experiments in shake flask cultures we could verify the observation of Sorokin (1995) that cell-free extracts of the organism do not show any sulphite oxidizing activity if the organism is cultivated without the addition of sulphite species (data not shown). Thus, in order to induce sulphite oxidase activity, sulphite has to be present in the cultivation medium. Furthermore chemical sulphite oxidation should be prevented. It is well-known (Cooper *et al.*, 1944; Van't Riet, 1979) that chemical oxidation of sulphite species is catalysed by transition metal ions (especially by Cu^{2+} but also by other ions).

Thus, one approach to prevent chemical sulphite oxidation is the addition of complexating agents to the cultivation medium. Our experiments with different concentrations of EDTA in the cultivation medium show that while the biomass concentration slightly decreases with increasing concentrations of the complexating agent the specific sulphite oxidase activity increases. In order to be able to verify the performance of each of the cultivation experiments we introduced a quality criterion, defined as the product of biomass concentration and specific sulphite oxidase activity. By means of this the main objective of the cultivation experiments is defined as the optimisation of the quality criterion. We observed the highest specific activity and the highest value for the quality criterion with a concentration of 51.3 µmol/l EDTA (tab. 1).

Table 1 - Comparison of generation times, biomass yield and specific enzyme activities in experiments with different EDTA concentrations.

EDTA conc. (µmol/l)	Generation time (h)	Biomass (g/l)		Spec. activity (U/mg)		Quality criterion
0	8.1	0.496	(100%)	6.0	(88%)	2.98
17.1	8.9	0.490	(99%)	6.2	(91%)	3.04
34.2	8.2	0.490	(99%)	5.8	(86%)	2.84
51.3	7.3	0.484	(98%)	6.8	(100%)	3.29

Our investigations on the influence of the acetate concentration on the cultivation performance in batch cultures show that there is a similar connection between acetate concentration and specific sulphite oxidase activity (tab. 2). While the biomass concentration increases slightly from 20 mmol/l to 50 mmol/l acetate concentration, the specific sulphite oxidase activity decreases drastically. Acetate concentrations higher than 50 mmol/l lead to a decrease in both biomass concentration and specific sulphite oxidase activity compared to the situation with 20 mmol/l. The data in table 2 shows also the highest value for the quality criterion at 20 mmol/l acetate concentration.

Table 2 - Comparison of generation times, biomass yield and specific enzyme activities for cultivation in 50%-Marine Broth medium with different amounts of sodium acetate.

Acetate concentration	Generation time (h)	Biomass (g/l)		Spec. activity (U/mg)		Quality criterion
20 mM	6.85	0.482	(94%)	6.9	(100%)	3.33
50 mM	6.45	0.513	(100%)	4.7	(69%)	2.41
80 mM	6.69	0.466	(91%)	4.5	(65%)	2.10
120 mM	6.65	0.417	(81%)	5.2	(76%)	2.17

Isolation of sulphite oxidase

For the isolation of the sulphite oxidase the following isolation steps were performed:

- centrifugation of the fermentation broth;
- ultrasonification of the cell pellet;
- second centrifugation;
- cation exchange of the supernatant of the second centrifugation;
- 30 kDa ultrafiltration.

The main part of this downstream procedure is the cation exchange step. The use of membrane adsorption techniques for this step offers in relation to conventional column chromatographic procedure some advantages, which lead to a better process performance, such as:

- lower manufacturing costs;
- non-diffusion controlled exchange kinetics so that higher fluxes are possible;
- easier handling in various module forms;
- easier upscaling.

Raising the separation efficiency by maximizing mass-transfer is the basic idea when using modified microporous membranes as the stationary matrix in liquid chromatography. Membranes can be converted into efficient adsorbers by attaching functional groups to the inner surface of synthetic microporous membranes. Affinity adsorption, ion exchange or immobilized metal affinity chromatography can be obtained by these membranes. Commercially available are membrane ion exchangers of strong acidic (sulfonic acid), strongly basic (quarternary ammonium), weakly acid (carboxylic acid), and weakly basic (diethylamine) types. A chelating membrane based on the iminodiacetate (IDA) group is applicable for IMAC. The membranes are available in products for lab and process scales. For process applications the modules and systems can be adapted to the special needs of the specific separation process to achieve optimal conditions. For production and large-scale application the Sartobind® Factor-Two Family (Sartorius, Germany) of membrane adsorber modules has been developed. The modules consist of a Sartobind® membrane reeled up like a paper roll to form a cylindrical module sealed at both ends with POM (polyoxymethylene) caps. For scaling up, the modules have areas between 0.12 m^2 and 8 m^2. The membrane adsorber technology has several major advantages compared to classical separation methods. Due to the membrane structure the binding of proteins is not limited by diffusional processes, therefore the loading and elution can be performed at very high fluxes resulting in very short cycle times. Compressibility of the membrane under normal operation conditions can be neglected, channeling can not occur, and the pressure distribution inside the modules is designed to have plug

flow through the module altogether leading to sharp breakthrough curves. Scale-up is very easy, materials and systems allow CIP (cleaning in place) and the validation of the process is made easier due to usage of standard products and validation service of suppliers. In figure 1 a typical chromatogramm for the isolation of sulphite oxidase from the crude extract is shown. For the elution of the bound protein the following gradient was used:

- Buffer A containing 20 mM acetate at pH 4.6;
- Buffer B containing 200 mM NaCl, 20 mM acetate at pH 4.6;
  - 0-5 min buffer A;
  - 5-20 min 0-20% buffer B;
  - 20-60 min 20-35% buffer B;
  - 60-70 min 35-100% buffer B;
  - 70-80 min buffer B.

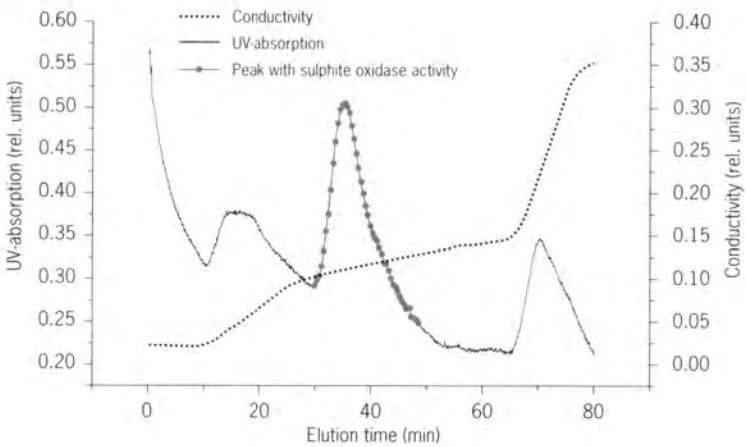

Figure 1
Cation exchange chromatography of crude cell extract of *Sulfitobacter pontiacus* for the isolation of sulphite oxidase using membrane adsorber (Sartorius, Germany).

While using the membrane adsorber in the downstream procedure described above, a highly purified sulphite oxidase can be isolated from the marine bacterium *Sulfitobacter pontiacus*. The specific activity of the enzyme in comparison with a commercial available sulphite oxidase from chicken liver is shown in figure 2.

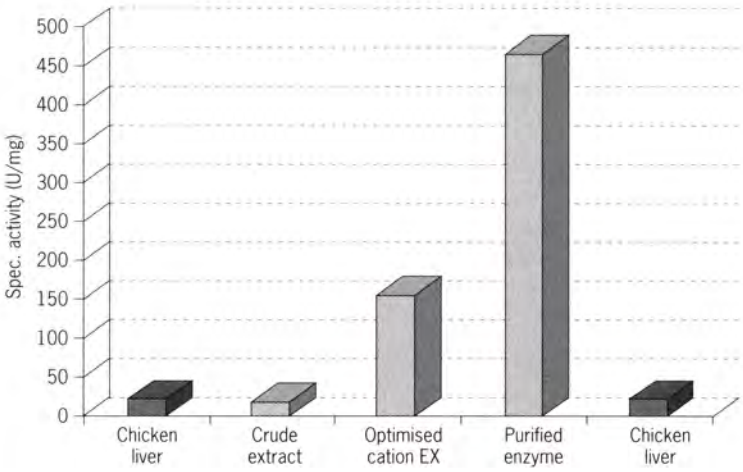

Figure 2
Comparison of specific activities of the sulphite oxidase during the downstream process and in comparison to a commercial available enzyme (isolated from chicken liver).

Conclusion

We investigated the influence of different parameters on the performance of batch cultivations of *Sulfitobacter pontiacus*. Our investigations were performed based on the commercially available medium Marine Broth 2216. An addition of EDTA to the cultivation medium proved very advantageous due to a reduction in chemical sulphite oxidation resulting in longer lifetimes of sulphite added to the medium as an inducing agent. Furthermore EDTA leads to a better growth, presumably due to a better bioavailability of ferric ions. The highest specific enzyme activities can be obtained under conditions with decreased or limited acetate concentrations. However a decrease in the concentration of the basic medium Marine Broth 2216 leads to a poorer cultivation performance. An addition of thiosulphate to the cultivation medium leads to reduced biomass concentrations and specific enzyme activities in comparison to cultivations without the addition of thiosulphate (data not shown). HEPES as a buffering agent proved advantageous due to the fact that the amount of sulphite to be added to the cultivations in order to induce the sulphite oxidase activity can be drastically reduced (data not shown). Regarding the influence of temperature and oxygen supply we observed that the best cultivation performance can be obtained with a temperature of 26°C in cultivation flasks equipped with to baffles. Using the membrane adsorber technique a very suitable downstream procedure for sulphite oxidase was established.

Acknowledgements
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SQDGs from *Porphyridium cruentum* inhibit the proliferation of human cancer cell-lines

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Abstract

Interest in the biological activity and pharmaceutical value of phytochemicals from marine origin has increased in the last decade. Many were found to possess *in vitro* selective toxicity against tumorigenic tissues and antiviral activity and therefore to possess a pharmaceutical potential. A crude SQDG sub-fraction isolated from the red microalga *Porphyridium cruentum* (SF) was found to inhibit *in vitro* the proliferation of murine immortalized L-929 cells and a panel of human solid cancer cells in a dose-dependant and cell-dependant manner via cytostatic and cytotoxic effects. The strongest activity was observed on human colon carcinoma DLD-1 cells and the weakest on human breast carcinoma MCF-7 cells. Compared to SQDGs from spinach used as a standard of plant SQDGs, SQDGs from *P. cruentum* were found markedly more active on all tested cell-lines due, in part, to a stronger ability to inhibit mammalian DNA polymerase pol-α, a key enzyme for DNA replication. This latter property might be related to the presence of large amounts of arachidonic and eicopentaenoic acid chains on the glycerol backbone. All these results suggested that SQDGs from *Porphyridium cruentum* might have a chemopreventive and/or chemotherapeutic potential. They justify new assays to isolate the most active compounds and evaluate their effectiveness in prevention and/or treatment of colon carcinoma

Introduction

There is growing interest for the red microalga *Porphyridium cruentum* due to its ability to excrete a sulphurized polysaccharide of industrial interest (Ramus *et al.*, 1989) and to accumulate large amounts of PUFAs, [especially arachidonic acid (ARA, 20:4 ω6) and eicosapentaenoic acid (EPA, 20:5 ω3) (Rebbolloso Fuentes *et al.*, 2000)] known to exert beneficial effects on human health. However, if the global fatty acid composition of *Porphyridium cruentum* and the influence of culture conditions on the concentration of active compounds are well documented (Rebbolloso Fuentes *et al.*, 2000; Alonso *et al.*, 1998), much less investigations have been done on the biological activities and pharmaceutical value of the individual class of lipids. Sulfoquinovosyldiacylglycerols

(SQDGs) have recently been reported to be DNA polymerase alpha inhibitors and therefore to have a potent pharmaceutical interest as anti-cancer agents (Ohta *et al.*, 2000). This study was designed to evaluate the ability of the crude SQDG fraction from *P. cruentum* (SF) to inhibit the proliferation of immortalized L-929 murine cells and a panel of human solid cancer cells comparatively to SQDGs from spinach (STD) used as a standard of plant SQDGs.

Material and methods

Biologicals and chemicals

Normal human fibroblasts were purchased from Biopredic International (Rennes, France). M4 Beu, a human melanoma cell-lines, was established in the laboratory of Dr. J.F. Doré (Inserm, National Institute for Health and Medical Research, Unit 218, Lyon, France). Breast cancer adenocarcinoma MCF 7, prostatic adenocarcinoma PC 3, lung carcinoma A 549, ovarian teratocarcinoma PA 1 and colon adenocarcinoma DLD-1 human cell-lines were purchased from the European collection of cell cultures (ECACC, Salisbury, UK). *Porphyridium cruentum* frozen cells were a gift from Dr G. Baudimant (Aquaartis, France).

Extraction and purification of algal SQDGs

The crude SQDG sub-fraction (SF, 75 mg) was obtained by (i) extraction of lipids (1630 mg) from the frozen algal biomass (50 g wet mass) with chloroform-methanol-water (Bligh & Dyer, 1959), (ii) precipitation of the dried residue with cold acetone (220 ml, - 20°C for 12 hrs), (iii) fractionation of the pellet (793 mg) into glycolipids (317 mg) by chromatography on silica gel (Bergé *et al.*, 1995), (iv) fractionation into sulfoglycolipids (132 mg) by ion exchange chromatography (Rouser *et al.*, 1976) and (v) purification by Folch wash (Folch *et al.*, 1957) and acetone precipitation. It was evaporated under nitrogen then stored at -18°C in C_6H_6-EtOH (1:1) under N_2 until use.

At all steps of purification, SQDGs were identified by TLC analysis on silica gel plates (F-254, Merck, Darmstadt, Germany) using migration in chloroform-methanol-water (65:25:4) and staining with orcinol reagent comparatively to an authentic standard isolated from spinach (STD).

Fatty acid analysis as FAME

To obtain FAME, a SF aliquot was evaporated under nitrogen and methylated by contact with methanol-sulphuric acid (98:2) in excess at 50°C for a night. After cooling, pentane (2 ml) and water (1 ml) were added and vortexed. The upper organic phases were collected and assayed using He as carrier gas on a Perkin Elmer autosystem fitted with a FID detector and a fused silica column (BPX-70, 60 m long, 0.25 mm i.d., 0.25 µm film thickness, SGE) programmed from 55°C (for 2 min) to 150°C at 20°C min^{-1} then to 230°C at 1.5°C.min^{-1}. Sample was injected with a split/splitless inlet and large volume injection system

(PSS) programmed from 55°C (for 2 min) to 350°C at 200°C.min^{-1}. FAMEs were identified by comparison with authentic standards and quantified using margaric acid (17:0) as internal standard. The fatty acid composition of STD was given by the manufacturer.

Cell proliferation and viability assays

Drug solutions and cell-lines cultures. SF and STD were dissolved in DMSO [final concentration in the culture medium 0.5% (v/v)] and immediately used for cytotoxicity assays. The maximal concentration tested (100 µg/ml for SF, 250 µg/ml for STD) corresponded to the maximal soluble concentration. Stock cell cultures and culture assays in 96 well plates were made as previously described (Barthomeuf *et al.*, 2001).

Cellular metabolic activity was performed using resazurin reduction test (RRT) according to O'Brien *et al.* (2000) with minor modifications (Barthomeuf *et al.*, 2001). Fluorescence was measured with an automated reader Fluoroskan Ascent FL^{TM} (Labsystems) at 530/590 nm. In the conditions of assay, fluorescence is proportional to the number of living cells in each well and IC_{50} value was calculated from the curve of concentration-dependent survival percentage. IC_{50} were calculated by regression analysis of concentration-response data and each value was acquired as the average of triplicate experiments.

Biomass content. Assay was carried out using Hoechst Dye 33342 according to Rago *et al.* (1990) with minor modifications (Barthomeuf *et al.*, 2001) by measuring fluorescence at 360/460 nm. In the conditions used, fluorescence is proportional to cell biomass and IC_{50} were calculated as above. Each value was acquired as the average of triplicate experiments.

Inhibition of DNA polymerase pol-α

Assay was carried out according to Hanashima *et al.* (2001) by incubating calf thymus DNA polymerase α (0.05 U in 50 mM Tris-HCl, pH 7.5) obtained as described by Tamai *et al.* (1988) with SF for 60 min at 37°C in a mixture (final volume 24 µl) containing 20 µM dTTP solution containing α-^{32}P-dTTP (1000 cpm/pmol), 15% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 µg/ml of poly(dA) and 10 µg/ml oligo(dT) (Rouser *et al.*, 1976). The enzyme was pre-incubated before assay on ice for 10 min with SF before addition of the enzyme-inhibitor component. Each value was acquired as the average of triplicate experiments.

Results and discussion

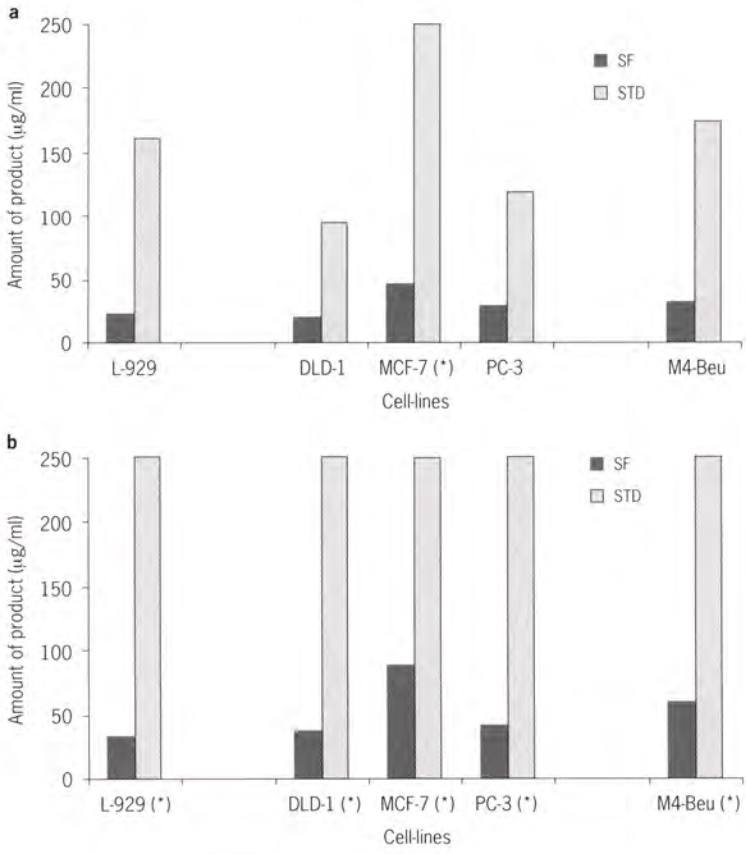
Extraction and fatty acid analysis of SQDGS from *P. cruentum*

The crude algal SQDG fraction (SF) represented ca 5% of total lipids. Results summarized in the table showed the respective composition in fatty acids of algal- and plant SQDGs. Once can be observed a clear difference in unsaturated fatty acid composition. SQDGs from plant contained

61% of linolenic acid isomers (C18:3n-3 et C18:3n-6), neither arachidonic acid (ARA, 20:4n-6), no eicosapentaenoic acid (EPA, 20:5n-3) whereas algal SQDGs were characterized by the presence of large amounts of PUFAs chains [ARA (36.8%) and EPA (16.6%)] and noticeable amounts of the mono-unsaturated fatty acid C16:1n-9 (10.5%) chains.

Compared ability of SQDGs from *P. cruentum* and plant SQDGs to inhibit the growth of solid cancer cell-lines

Algal SQDGs (SF) were assayed for antiproliferative activity comparatively to STD, on murine immortalized cells L-929, and a panel of human solid cancer cells (colon adenocarcinoma DLD-1, breast adenocarcinoma MCF-7, prostatic adenocarcinoma PC-3 cells and malignant melanoma cells M4-Beu) by assaying both the residual metabolic activity (RTT test) and the residual cell biomass (Hoechst Dye 33342 test) of cultures submitted to a 48 h continuous treatment with drugs. Results (fig. 1) unambiguously demonstrated that (i) SQDGs from *P. cruentum* inhibited the proliferation of all tested cell-lines in a dose-dependant manner and (ii) were markedly more active than SQDGs from plant in all experimental conditions. IC_{50} for growth inhibition were dependant on the cell-line (fig. a) but always weaker than IC_{50} values for cell death (fig. b),



Antiproliferative effects of SF and STD on murine immortalized cells L-929, human colon carcinoma cells DLD-1, human breast carcinoma cells MCF-7, human prostatic carcinoma cells PC-3 and human malignant melanoma M4-Beu. IC_{50} values for residual biomass (Hoechst Dye 33342 test) a) and for residual cellular metabolic activity (RTT test) b) after 48 h continuous contact with drug. Results are average of 3 experiments. (*) IC_{50} more than 250 µg/ml.

suggesting that growth inhibition was related to cytostatic and cytotoxic effects. The strongest activity was observed on colon adenocarcinoma DLD-1 and the weakest on breast MCF-7 cells.

Compared ability to inhibit DNA polymerase pol-α

To determine if the stronger antiproliferative effect of SF could be related to a stronger ability to inhibit DNA polymerase pol-α, each fraction was tested for this property using an enzymatic method previously used by Hanashima *et al.* (2001) to assess IC_{50} values of synthetic SQDGs containing glucose as sugar moiety. IC_{50} values for enzymatic inhibition were found equal to 378 µg/ml for SF and more than 1000 µg/ml for STD. DNA polymerase pol-α is known to be involved in early step of DNA replication and, therefore, to be a key enzyme for cell division. The stronger inhibition of DNA polymerase activity by SQDGs from *P. cruentum* compared to SQDGs from spinach could therefore partly explain their stronger antiproliferative activity in cell under proliferation. Based on reported data (Ohta *et al.*, 2000; Hanashima *et al.*, 2001) demonstrating a relationship between the number, size and unsaturation degree of the fatty acid chains linked on the glycerol backbone and the ability of synthetic SQDGs to inhibit DNA polymerase pol-α, this property might be related to the presence of ARA and EPA chains in place of linolenic acid chains. This hypothesis is in agreement with the observations of Hanashima *et al.* (2001) who reported that SQDGs with longer carbon chains were more effective for inhibition of DNA polymerase activity than those with shorter carbon chain. However, as IC_{50} for inhibition of enzyme activity being about 10 times higher than IC_{50} for inhibition of cell proliferation, it is clear than other mechanisms than inhibition of DNA polymerase pol-α are involved in the "anticancer" activity of SQDGs from *P. cruentum*. Complementary experiments are under way to identify some of these mechanisms.

Fatty acid composition of SQDGs fractions.

Fatty acids	SF* (µg, %)	STD** (µM, %)
C16 :0	26.1	28
C16 :1n-9	10.5	
C18 :0	0.75	2
C18 :1n-9 cis	0.55	
C18 :1n-7	0.50	3
C18 :2n-6 cis	4.0	6
C18 :3n-6	0.5	
C18 :3n-3	0.3	61
C20 :2n-6	0.4	
C20 :3n-6	2.9	
C20 :4n-6	36.9	
C20 :5n-3	16.6	

* SQDG from *P. cruentum*, ** SQDGs from spinach (values reported by the manufacturer).

In conclusion, these preliminary results demonstrated that SQDGs from the red alga *Porphyridium cruentum* effectively inhibit *in vitro* the proliferation of human cancer cells. They show that inhibition is (i) dose-dependant, (ii) differs with the type of cancer cells, (iii) is related to both cytostatic and cytotoxic effects and (iv) is markedly more important than that of SQDGs from plant used as a standard. This stronger effect could be partly related to a stronger ability of SF to inhibit DNA polymerase pol-α activity which might partly due to the presence of arachidonic and/or eicosapentaenoic acid chains at C1 and/or C2 of the glycerol backbone. Activity against colon carcinoma DLD-1 justifies new evaluations. New experiments are under way to isolate the most active compounds, determine their structures and identify the mechanisms involved in activity.

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Modelling of growth and product formation of *Porphyridium purpureum* under defined conditions

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Abstract

In this contribution both experimental data and simulations of growth rate and product formation of the unicellular microalgae *Porphyridium purpureum* are presented. The mathematical model has been developed for a better understanding of the processes in production plants. This is achieved on one hand by the usage of a scale-up model, on the other hand by the inclusion of the dynamic metabolic behaviour of the different macro-molecules under changing conditions. The mathematical simulation model has been refined with the results of several cultivations that have been carried out in a new photo-bioreactor which allows for the study of microalgal kinetics under defined illumination conditions.

Material and methods

Reactor

The used photo-bioreactor is a stirred reactor with a volume of three litres. Light is generated in a light source arranged externally and distributed by means of optical fibres on an internal draft tube which also serves as an irradiation element. Any focusable light source can be applied by using a shutter module. Therefore, it is possible to generate light fluctuations in a wide range of frequencies or to change the light quality by means of spectral filters placed in front of the optical fibre.
All cultivations were performed in turbidostate mode. In order to control the medium flow rate, the optical density was calculated on-line by using a transmission cell connected in bypass. By this means the biomass concentration can be kept at a defined value allowing a good comparability of the measurement data. The influence of different transient light intensity changes like step response and light-dark-cycles (in the range ms to s) has been investigated, the results were integrated into the mathematical model.

Off-line cultivation parameters

The different off-line cultivation parameters like the optical density, the pigment concentration, the protein concentration or the polysac-

charide concentration were obtained with the help of spectrometer measurements.

Mathematical model

The mathematical modelling of a photo-bioreactor requires to know the coupling between the metabolism of microorganisms and the physical phenomena of light transfer inside the culture medium and the fluid dynamics of the reactor. To describe the specific growth rate and the product formation, a structured model has been developed. By definition, a structured model considers the internal structures and the states of a cell. But even the simple bacterium undertakes over 2000 enzymatically catalyzed reactions. So simplification is necessary.
The developed mathematical model is build up with the help of the program Matlab/Simulink.

Results of a cultivation

Cultivation results with the microalgae *Porphyridium purpureum* are shown in figure 1. For this cultivation the 150 W light source and an acrylic glass cylinder have been used. A growth rate of $\mu = 0.42/d$ was attained in the exponential growth phase using artificial sea water medium at 20°C, pH = 7.6. After the batch phase, the continuous cultivation was started. The biomass concentration stays at the constant level of approx. 0,2 g/l.

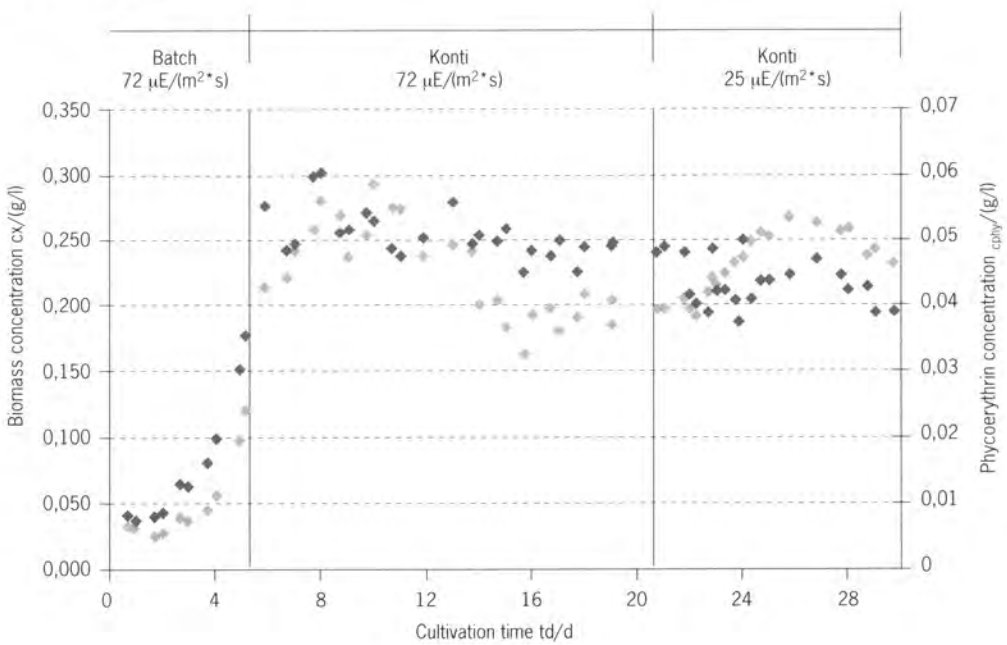


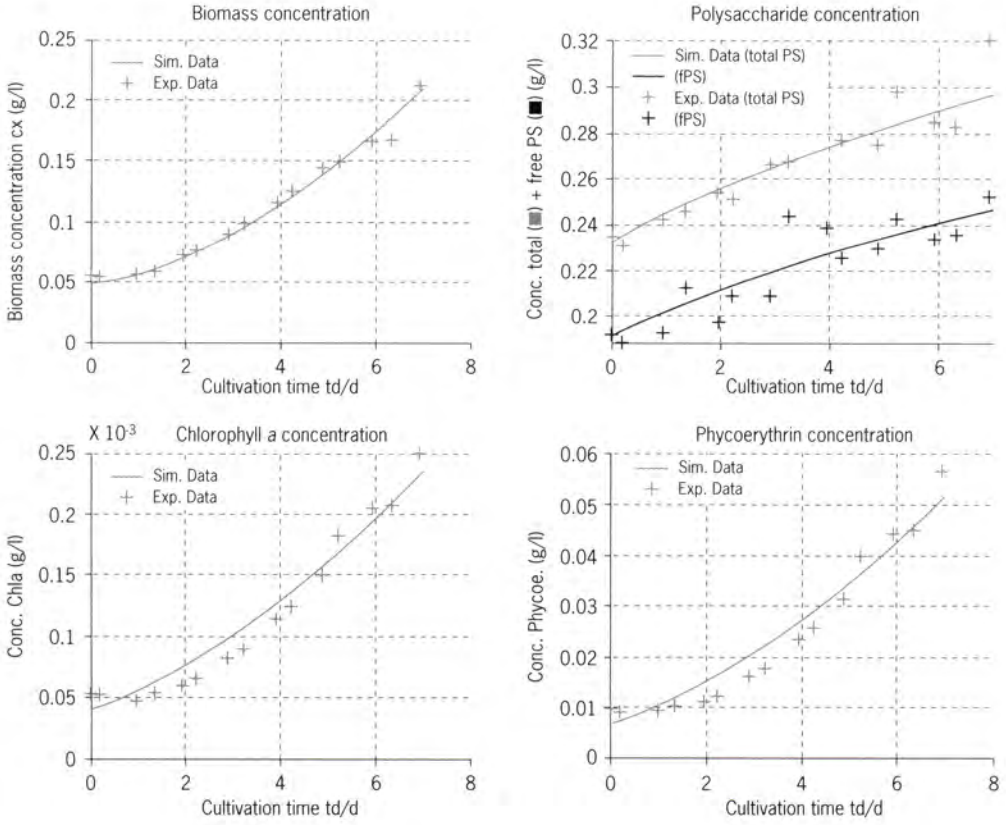
Figure 1
Growth of the microalgae *Porphyridium purpureum* and the concentration of the pigment phycoerythrin is represented. The culture medium is artificial sea water medium illuminated with 150 W light source and acrylic glass cylinder as light emitting tube.

The influence of two light intensities was studied. In the first phase, the light intensity was 72 $\mu E/m^2.s$, the phycoerythrin concentration decreases, and the specific production rate was r_{PE} = -0,058 (g/g.d). The pigment production increases in the second phase of the cultivation because the light intensity was decreased. All the obtained off-line or on-line information can be used to develop the mathematical model.

Results of the simulation

The results of the simulated cultivations are represented in comparison with the determined measured data. In the next picture, the different results are graphically represented for a batch cultivation with the light intensity 10 $\mu E/m^2.s$. In the figure 2, both the simulated (continuous line) and the measured data (crosses) for the different macromolecules namely the biomass, the total and the free polysaccharide concentrations, the chlorophyll a and the phycoerythrin concentrations are represented. The simulated data show a very strong correlation with the obtained experimental data.

Figure 2
Results of simulated and experimental data for a batch cultivation with 10 $\mu E/m^2.s$ for the microalgae *Porphyridium purpureum* and its products.



But not only batch but also continuous cultivation can be simulated with the developed mathematical model. So in the figure 3, the results of the transition between batch and continuous cultivations at the same light intensity as the batch cultivation are shown.
The developed mathematical model can be used for a lot of different possible light intensities.

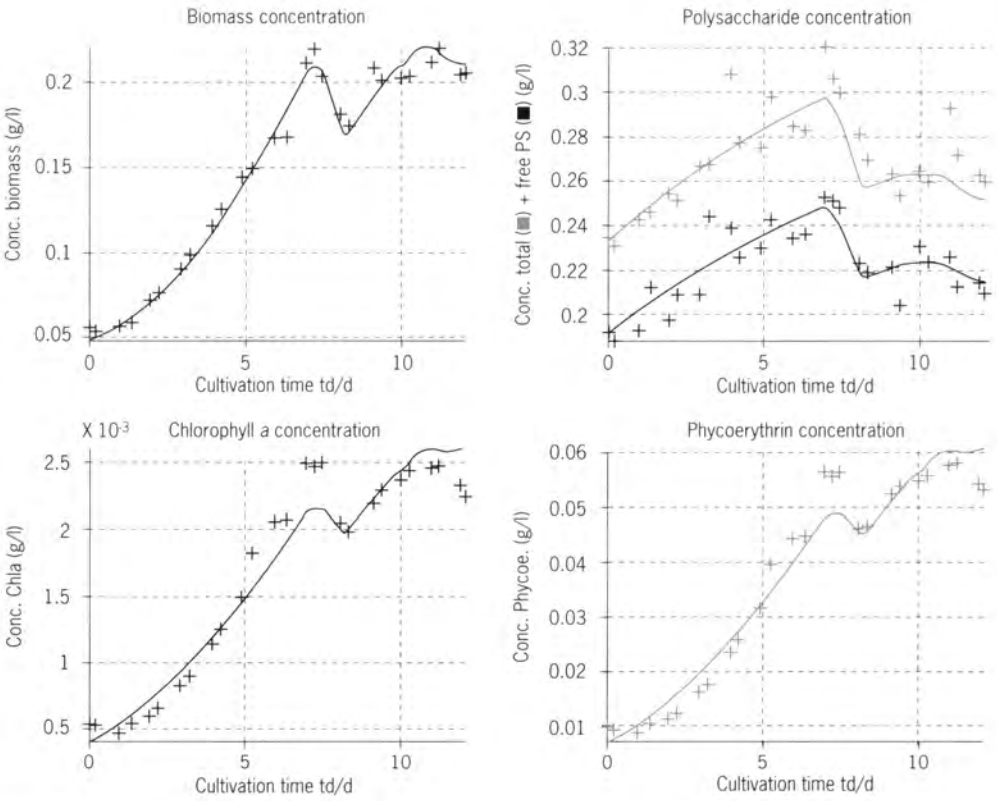


Figure 3
Results of simulated and experimental data for a batch-continuous cultivation with 10 µE/m².s for the microalgae *Porphyridium purpureum* and this products.

Conclusion

In the further work, it is planned to separate the mathematical model in three different levels: the reactor level, the level of the functional macromolecules and the level of the metabolic network. The difference between the two last levels is the time constant. The time constant of the functional macromolecules is about hours or days. In opposite of this the time constant of the metabolic network is in the range of seconds. But the different interactions between these two levels should be described.

Output variables of the model are among others the commercially interesting macro-molecules of the microalgae, e.g. polysaccharides, pigments and polyunsaturated fatty acids. This enables a reliable forecast of the specific growth rates of these products for the production in a larger scale.

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Long-term production and extraction of carotenoids from the living cells of the microalga *Dunaliella salina*

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Abstract

A new method for the simultaneous production and extraction of carotenoids from *Dunaliella salina* was developed. Growth of the cells was performed under low light intensity and then the cells were transferred to a production and extraction bioreactor, which was illuminated at a higher light intensity. Biocompatible organic phase was used in the production bioreactor. Mixing and extraction in the second bioreactor were performed by re-circulation of the organic phase. For studying the effect of the mixing, solvent re-circulation, rate on the cells two different mixing rates were applied.

The results indicated that mixing rate has significant influence on the rate of carotenoid extraction. The higher the mixing rate the higher the extraction rate was achieved. Furthermore, the results showed that growth of *Dunaliella salina* in the presence of a biocompatible organic phase and high light intensity is very slow. However, the cells keep β-carotene production for a long period in the presence of the biocompatible solvent. β-carotene extraction efficiency by the biocompatible solvent is 56.5%. The rest of carotenoids remains inside the biomass which can be dried and utilized as carotenoids rich biomass. The relative concentration of carotenoids over the chlorophyll inside the cells was 35 g/g.

Introduction

Carotenoids are widely used in food, pharmaceutical and cosmetic industries as pro-vitamin A, antioxidant and colorant agents. Recently, a great demand of carotenoids has been answered by industry, both by synthetic production and by preparation of this compound from natural sources. Consumers have currently a critical attitude about synthetic products and they show a clear preference for natural products. Increasing demand for natural carotenoids has resulted in growing interest in extracting β-carotene from different sources (Vega *et al.*, 1996). Estimated market size for β-carotene is 10-100 (>25) $t.year^{-1}$ and its price is >750 US$ Kg^{-1} (Pulz *et al.*, 2001). *Dunaliella salina*, the main source of the natural β-carotene in the market, is a unicellular green microalga

that can produce and accumulate β-carotene under stress conditions. High light intensity, high salinity and nutrient deficiency are examples of these stress factors. β-carotene can be prepared by spray-drying of algal biomass and sold in the form of β-carotene-rich biomass tablets or capsules. It can also be separated from the algal cells by extraction with organic solvents or edible oils (Leach *et al.*, 1998).

Hejazi *et al.* (2002) studied the possibility of application of a two-phase bioreactor for the production and extraction of β-carotene by *Dunaliella salina* with retention of the viability of the cells. They showed that *Dunaliella salina* can stay active and viable in the presence of organic solvents with log P > 6. It was also shown that high purity β-carotene can be extracted due to a preference for the extraction of β-carotene over chlorophyll by the biocompatible solvent phase. Ben-Amotz (1993) explained that from physiological point of view growth and β-carotene production are two different stages in the life cycle of *Dunaliella salina*. They achieved higher productivity by application of two step cultivation of *Dunaliella salina*.

The objective of this paper is to develop a new method for the long-term and simultaneous production of carotenoids from microalgae on the basis of the former report of Hejazi *et al.* (2002). To obtain higher productivity the growth phase and production and extraction phase were separated. *Dunaliella salina* and β-carotene have been chosen as a model for extraction of lipophilic compounds from microalgae.

Material and methods

Organism and medium

Dunaliella salina (CCAP 19/18) was grown in the culture medium containing 1M NaCl, 10 mM KNO_3 and 1 mM $NaH_2PO_4.2H_2O$. The pH of the medium was adjusted to 7.5 by some drops of a 3 M HCl. The medium was sterilized at 121°C for 40 minutes before inoculation. To avoid precipitation, the phosphorous was autoclaved separately and solid carbon sources ($NaHCO_3$) was put in the oven at 120 °C over night and then mixed with the sterilized water.

Experimental setup

Growth and β-carotene production by *Dunaliella salina* were carried out in two different bioreactors. Growth was carried out in a bubble column bioreactor at low light intensity (450 mmol m^{-2} s^{-1}). Then, the cells were transferred to the second bioreactor when the concentration reached $1.6 * 10^9$ cell.l^{-1}. In the second bioreactor, which was a flat panel bioreactor with depth of 2.5 cm, the cells were diluted two times by the fresh culture media. β-carotene production and extraction were performed in the second bioreactor at higher light intensity (1200 mmol m^{-2} s^{-1}). Dodecane, which its biocompatibility for the cells of *Dunaliella salina* had been already approved (Hejazi *et al.*, 2002), was applied as organic phase in the second bioreactor. The bioreactor contained 80%

of aqueous phase and 20% of organic phase. Both mixing of the culture media and β-carotene extraction, in the production bioreactor, were carried out by re-circulation of the organic biocompatible solvent through the aqueous phase. The temperature was controlled at 26±1°C.
The experiment was performed in two steps. The first step was a short-term experiment for determination of the possible effect of the different mixing, solvent re-circulation, rates on the cell population and carotenoids production and extraction. Two different runs with two mixing rates of 150 and 200 $ml.min^{-1}$ were performed. The second step was a long-term experiment and was designed on the basis of the results of the first step. In the second experiment, the organic phase was replaced 3 times during the experiment when the concentration of carotenoids was about 110 $mg.l^{-1}$.

Cell counting

The cell number was determined by counting the cells using 0.1 mm deep counting chamber (Neubauer improved). The optical density of the culture media was also determined spectrophotometrically (Spectronic, 20 GENE SYS).

Carotenoids and chlorophyll determination

β-carotene and chlorophyll concentrations were determined spectrophotometrically. For that purpose, standard curves for β-carotene and chlorophyll *a* concentrations were made by us. β-carotene and chlorophyll *a* concentrations were plotted as a function of absorbance and calibration equations were determined by linear regression.

Results and discussion

Short-term experiment

Growth of algal cells

The growth step was performed in the first bioreactor and after reaching certain cell concentration the cells were transferred to the second bioreactor. The main goal in the second bioreactor was production and extraction of carotenoids, however for studying the effect of the differed mixing rates on the cell population, growth of the cells was followed for one week in the second bioreactor. Results indicated that application of different mixing rates, 150 and 200 $ml.min^{-1}$, does not have a significant effect on the cell population (fig. 1).

Carotenoids production and extraction

Carotenoids content of the cells (aqueous phase) and organic phase was determined for both of the bioreactors. Results indicated no significant differences on the carotenoids concentration in the cells, however carotenoids content of the organic phases was significantly different. Extraction rate was 3 times higher when the higher mixing rate was applied (fig. 2).

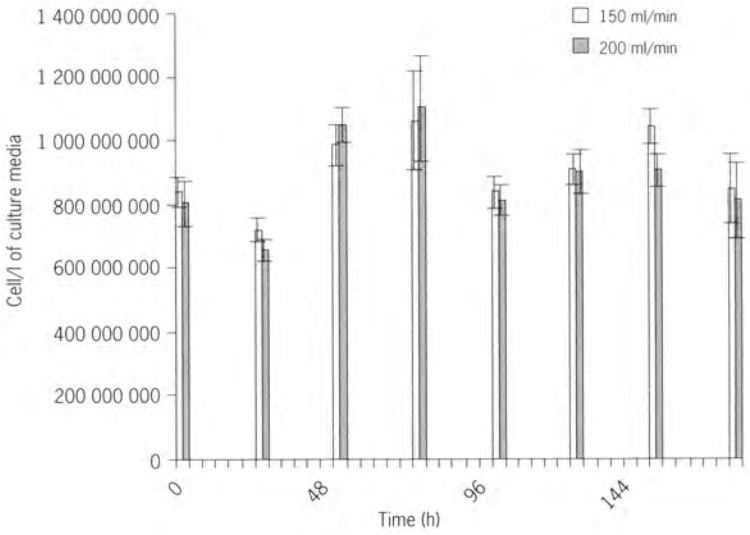

Figure 1
Cell population response to the different mixing rates.

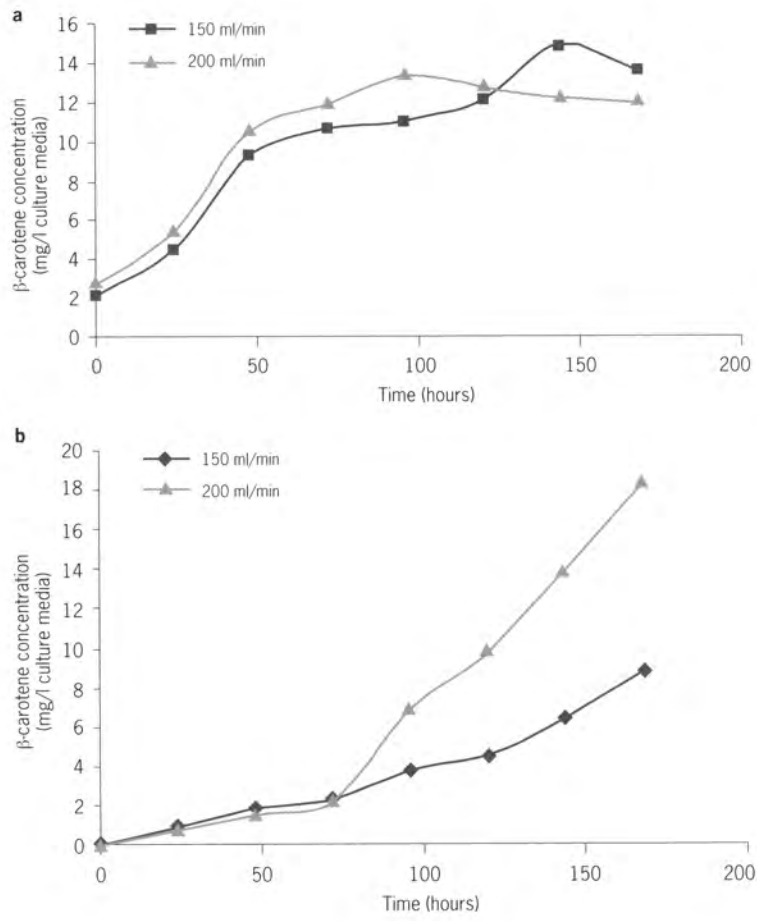

Figure 2
Carotenoids content of the aqueous (a) and organic (b) phases in response to different mixing rates.

Long-term experiment

On the basis of the results of the short-term experiment, the long-term experiment was performed. All the conditions were the same and 200 $ml.min^{-1}$ was chosen as solvent re-circulation rate.

Growth of algal cells

Again the cells were grown in the growth bioreactor and after getting certain concentration the cells were transferred to the production and extraction bioreactor. Cell growth was followed by regular sampling and direct counting of the cells under the microscope. In the first week of the experiment fluctuation in the cell concentration was observed. After that, the cells showed very slow growth and in the last week of the experiment the cell population stayed almost constant (fig. 3). It is already known that the cell growth is limited when they produce carotenoids (Ben-Amotz, 1993), but it seems that presence of an organic biocompatible phase has extra effect on the growth of the cells at higher light intensities.

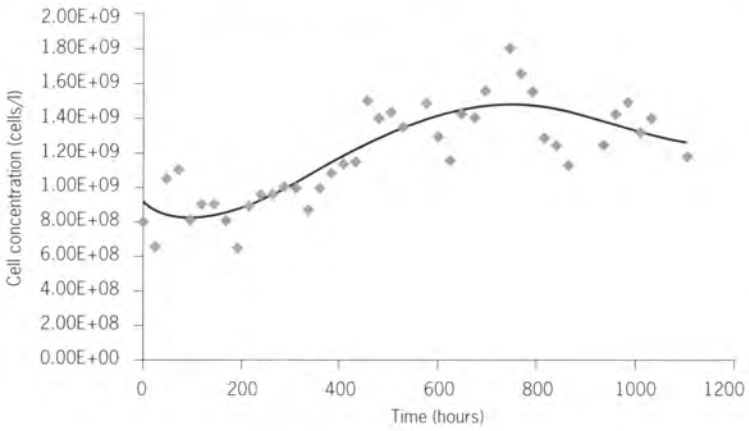

Figure 3
Growth of *Dunaliella salina* in the presence of organic biocompatible solvent.

Carotenoids production in the presence of organic phase

Figure 4 shows carotenoids content of the bioreactor (both aqueous and organic phases) as a function of time. It shows that not only the cells continue carotenoids production for long time in the presence of organic phase but also carotenoids content of the cells increases by the time.

Chlorophyll production by the cells

In contrast to the carotenoids production by the cells chlorophyll production showed a different pattern (fig. 5). The chlorophyll content of the cells decreased in the first week when the cells did not grow. After one week when the cells started to grow and the cell concentration increased, the cells started to produce more chlorophyll. After 600 hours when the cell growth stopped chlorophyll concentration decreased

as well and it reached until 1.43 pg per cell. On the other hand, the carotenoid content of the cell increased by the time and it reached to 51 pg per cell. The ratio of carotenoid over chlorophyll reached from 0.6 to 35 (fig. 6). It is 3.5 times higher than the ratio which was reported by Ben-Amotz in 1993.

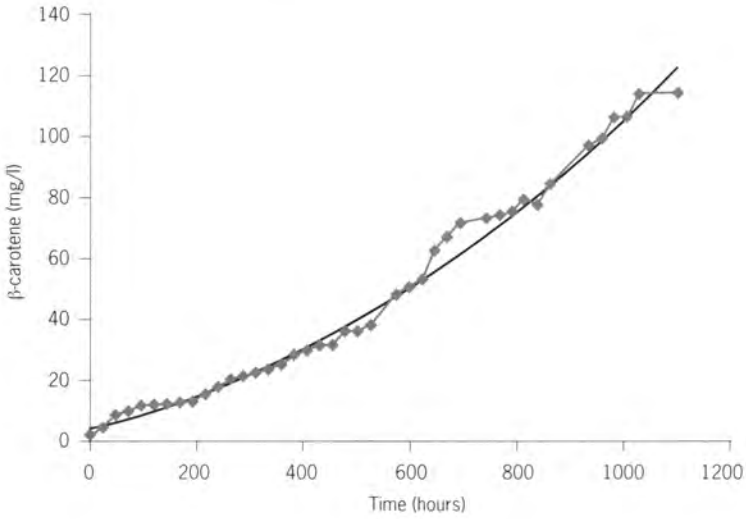

Figure 4
Total volumetric production of carotenoids in the presence of organic phase.

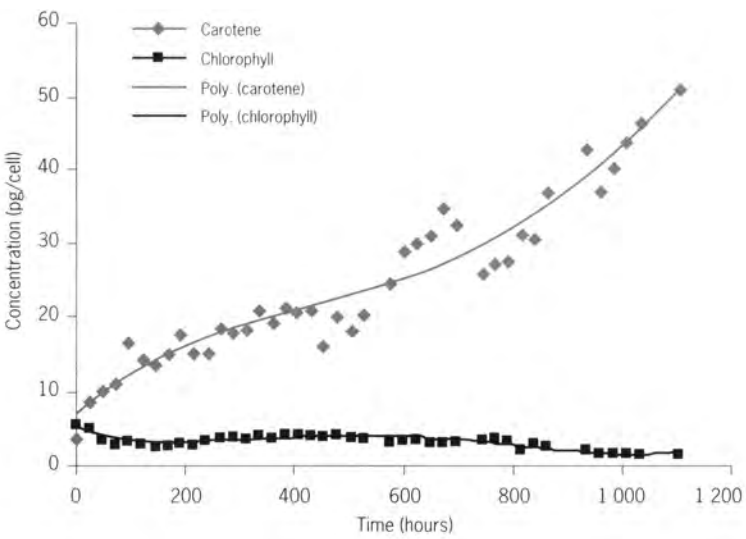

Figure 5
Carotenoids and chlorophyll content of the cells as a function of time.

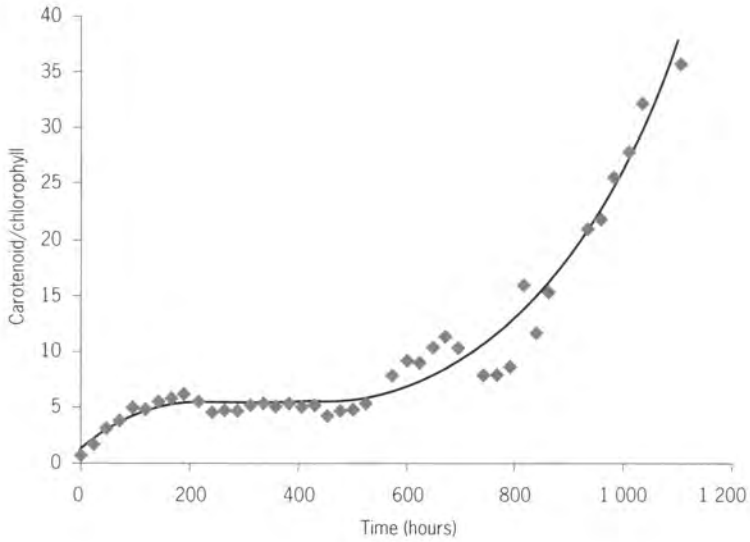

Figure 6
Carotenoid/chlorophyll as a function of time.

Carotenoids extraction in the organic phase

Figure 7 shows the concentration of extracted carotenoids per liter of organic phase. As already mentioned in the material and methods section only 20% of the total volume of the bioreactor was organic phase. It means that real extracted amount of the carotenoids was 65 mg. Figure 4 shows that total carotenoids production was 115 mg. Then extraction efficiency is 56.5%. The extracted part contains high purity carotenoids, which can directly be used after one simple separation operation. The rest of carotenoids remains inside the biomass which can be dried and utilized as carotenoids rich biomass.

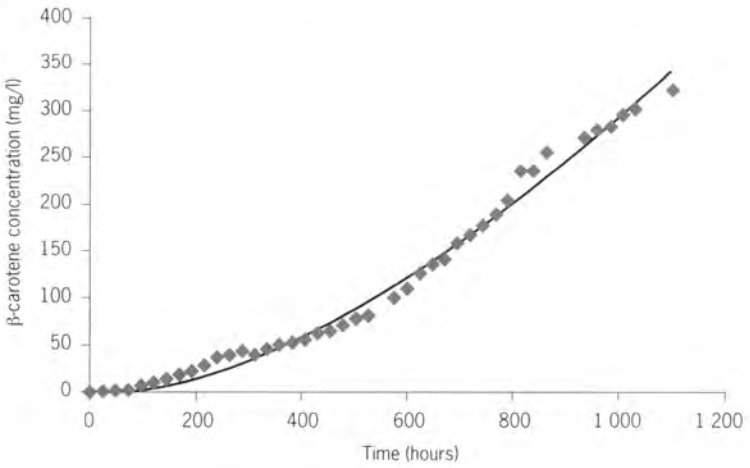

Figure 7
Carotenoids concentration in the organic phase.

Conclusion

All the common methods are on the basis of harvesting the cells, and application of multi-step extraction processes to extract and refine the β-carotene. But we showed that the cells stay viable and keep β-carotene production for a long term in the presence of biocompatible organic phase.

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Unsaponifiable matter of the cultured diatom *Odontella aurita*: phytol and sterols as major components

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Abstract

Unsaponifiable matter from lipids of the cultured diatom *Odontella aurita* was studied. Phytol occurred as major component accounting for up to 40% in the unsaponifiable matter (0.15% dry wt). Ten components of the sterol fraction (0.08% dry wt) were found and identified including ergosta-5,22-dienol (up to 52.5% of the sterol fraction), ergosta-5,24(24')-dienol (up to 23.5%) and the unusual 23,24-dimethylcholesta-5,22-dienol (13.7%). Hydrocarbons, including carotenoids and triterpenoids, accounted for 0.05% (dry wt). Tocopherols were also present as minor components.

Introduction

The diatom *Odontella aurita* is known for the high levels of polyunsaturated fatty acids, particularly 20:5 (n-3), EPA. Diatoms are a food source for aquaculture and have applications in the field of cosmetics. Despite the importance of this diatom, there are only few studies on sterols or other compounds (Orcutt & Patterson, 1975; Volkman *et al.*, 1980; Shifrin & Chisholm, 1981; Braud, 1998). Microalgae generally form the base of aquatic food webs. They are an alternative fatty acid source to oilseed and fish (n-3) and (n-6). Fatty acids are a pharmacologically interesting group of valuable biomaterial. It is well-known that the production and storage of lipids by algal cells is regulated by environmental factors such as light intensity, nutrient stress and temperature. Most of the studies have been concerned with total lipids or only fatty acids. Few studies have been investigated the composition of the unsaponifiable matter.

The aim of this work was to perform a comparative study of the unsaponifiable matter obtained from frozen and freeze-dried biomasses regarding the composition of sterols and other components such as terpenic alcohols, hydrocarbons, anti-oxydants occurring in the diatom *Odontella aurita* cultured in defined conditions.

Material and methods

Samples of biomass (frozen and freeze-dried) of *Odontella aurita* were prepared by Innovalg Co (Bouin, France) from cultures in pilote tanks. The algal biomass was ground with dichloromethane-methanol and steeped twice in that mixture (2:1 and then 1:2, v/v, respectively) at room temperature (fig. 1). The combined extracts yielded the crude total lipids. Alkaline saponification (KOH/EtOH, 2 h under reflux) allowed to obtain fatty acids and unsaponifiable matter. Fatty acids were analysed as methyl esters and N-acyl pyrrolidides by GC/MS. Aqueous phase was extracted three times with hexane and then acidified. The fatty acids obtained were converted into methyl esters by reaction (30 min under reflux) with methanolic hydrogen chloride. N-acyl pyrrolidides were prepared by direct treatment of methyl esters with pyrrolidine/acetic acid (10:1, v/v) for 2 h under reflux and purified by TLC on 0.5 mm silica gel layers, using hexane/diethyl ether (1:2, v/v).

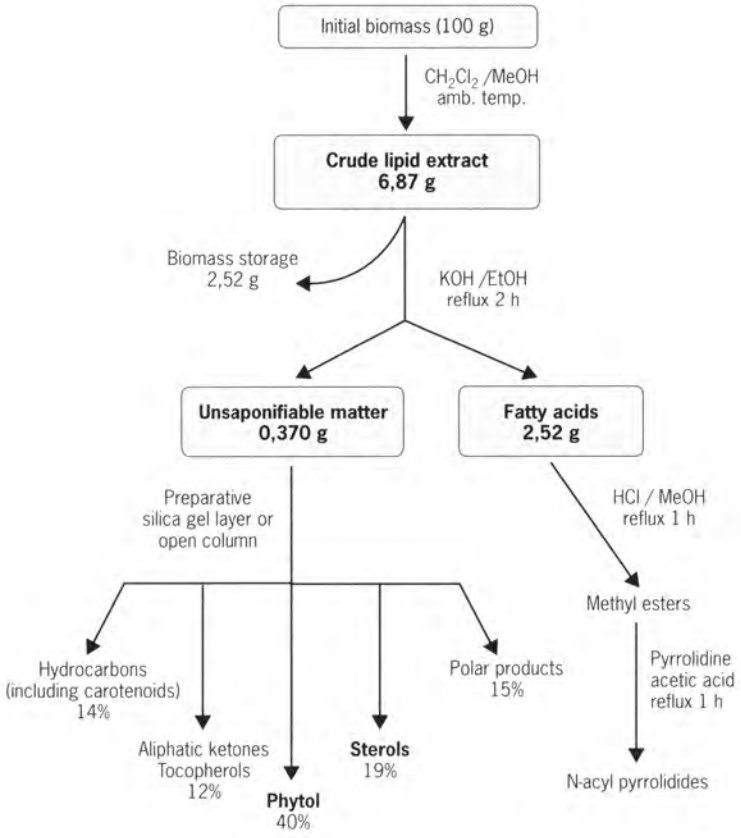


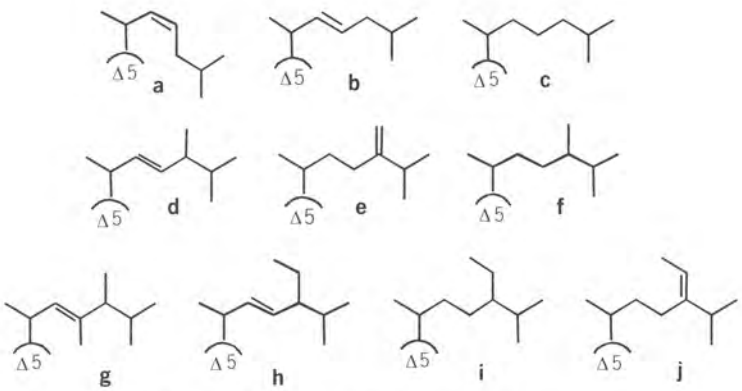
Figure 1
General procedures for the saponification and the determination of the composition of unsaponifiable matters from *Odontella aurita* (example of the freeze-dried biomass).

The unsaponifiable matter was subjected to a preparative layer chromatography (on 0.5 mm plates), and to a silica gel column chromatography and ten bands were scrapped off. The total sterols (recrystallized from methanol) were acetylated with acetic anhydride-pyridine (dried on potassium hydroxyde) for 20 h at room temperature. Total sterols were analysed in free form or as acetates by GC/MS.
GC/MS experiments were performed on a Hewlett-Packard HP 5890 gas chromatograph using a 30 m x 0.32 mm id fused silica capillary column coated with HP-1 (0.25 mm phase thickness, helium as carrier gas) coupled with a HP 5989A mass spectrometer (EI 70eV) equipped with a HP 9000 integrator. Column temperature was programmed for fatty acid methyl esters and pyrrolidides from 180 to 310°C at 3°C.min^{-1}, and for sterols and steryl acetates from 170 to 300°C at 3°C.min^{-1}.
Some compounds such as carotenoids, tocopherols, triterpenoids were identified from their mass spectra by comparison with commercial standards.

Results and discussion

The two unsaponifiable matters studied (see figure 1 for procedures) have very similar biochemical compositions. Nevertheless, more hydrocarbons (carotenoids) in unsaponifiable matter from freeze-dried biomass than in those from the frozen biomass were found. 9.3% of unsaponifiable matter was obtained from the freezed biomass and 8.5% from the freeze-dried biomass.
Phytol occurred as a major component in both unsaponifiable matters accounting for about 40% (weight). Phytol mainly originates from chlorophyll and is used for industrial preparation of vitamins E and K_1. Production of phytol from *O. aurita* in these conditions can be estimated at 1.4 g/kg (dried biomass).
Various hydrocarbons including carotenoids and triterpenoids, fatty ketones, tocopherols, aliphatic alcohols were found as minor compounds. Carotenoids are used as non-toxic dye in food and cosmetic preparations. The anti-oxydant properties of β-carotene have received some attention due to its potential application in clinical and nutritional fields. Tocopherols (vitamin E) function as radical-chain terminators and highly efficient singlet-oxygen scavengers.
The sterols accounted for about 20% of the unsaponifiables, including three major compounds: ergosta-5,22-dienol (52.5% of the total sterol fraction), 24-methylene-cholesterol (23.7%) and the unusual 22,23-dimethylcholestadienol (13.7%). It should be noted that 24-methylene-cholesterol occurred at unusual high levels. The latter sterol, known as a precursor of gorgosterol, has been already observed in *Biddulphia sinensis* at lower amounts: 0.9-2.9% (Orcutt & Patterson, 1975). Ergost-8(9)-enol previously found in *B. aurita* was not detected as well as any Δ7 sterol (Volkman *et al.*, 1980).

Major fatty acids were myristic, palmitic, palmitoleic, vaccenic, 16:4 (n-1), 16:3 (n-4), 20:5 (n-3) EPA, 22:6 (n-3) DHA.



d : Ergosta-5,22-dienol **e** : Ergosta-5,24(24')-dienol
g : 23,24-Dimethylcholesta-5,22-dienol

Figure 2
Side chains of the Δ5 sterols occurring in *Odontella aurita*. Sterols d, e and g were found as major components: 52.5, 23.7 and 13.7% respectively. The other sterols were present at less than 5%.

Acknowledgements
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Primmorphs from sponges as bioreactors for a sustainable production of bioactive compounds

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Abstract

Sponges (phylum Porifera) are a rich source for the isolation of biologically active and pharmacologically valuable compounds with a high potential to become effective drugs for therapeutic use. However, until now, only one compound could be introduced into clinics because of the limited amounts of starting material available for extraction. To overcome this serious problem in line with the rules for a sustainable use of marine resources, the following routes can be pursued: first, chemical synthesis, second, cultivation of sponges in the sea (mariculture), third, growth of sponge specimens in a bioreactor, and fourth, cultivation of sponge cells *in vitro* in a bioreactor. The main efforts to follow the latter strategy have been undertaken with the marine sponge *Suberites domuncula*. A sponge cell culture was established after finding that single sponge cells require cell-cell contact in order to retain their telomerase activity, one prerequisite for continuous cell proliferation. The sponge cell culture system, the primmorphs, comprises proliferating cells which have the potency to differentiate. Future strategies to improve the sponge cell culture are discussed.

Introduction

Members of the phylum Porifera (sponges) contain - perhaps besides the Bryozoa - the largest number of bioactive compounds among all Metazoa (reviewed in: Sarma *et al.*, 1993; Proksch, 1994; Faulkner, 2000). However, despite the large number and the high variety of structurally different natural products only very few of these marine secondary metabolites have been tested in clinical trials. So far, arabinofuranosyladenine [ara-A; isolated from the gorgonian *Eunicella cavolini* (Cimino *et al.*, 1984)], a derivative of ara-U, isolated from the sponge *Cryptotethya crypta* (Bergmann & Feeney, 1951) is the only secondary metabolite (Cohen, 1963) which has been approved for human appli-

cation in clinics; it displays potent anti-herpes virus activity (Müller *et al.*, 1975, 1977). Some other sponge metabolites, including avarol (Minale *et al.*, 1974; Müller *et al.*, 1985a,b) have reached clinical evaluation.
It is clear that the limited availability of large quantities of defined starting material from a certain sponge species for extraction of bioactive compounds is the major cause for the low attractiveness of such secondary metabolites for commercial exploitation. Four routes can be followed to obtain greater amounts of sponge secondary metabolites.
- First, chemical synthesis; this approach has successfully been undertaken (reviewed in: Faulkner, 1996) but, in numerous cases, requires many steps with only low yields;
- Second, cultivation of sponges in the sea, in mariculture;
- Third, cultivation of sponge specimens in a bioreactor, as has been investigated during the last years (Osinga *et al.*, 1998). In this field considerable progress has been made in the laboratories of (Brümmer to be published) and in our group (Le Pennec *et al.*, submitted);
- Fourth, production of secondary metabolites in bioreactors using sponge cells in culture (in analogy to the production of bioactive compounds from bacteria and fungi); this is a route that indicates progress. The first successful approach to show that sponge cells can proliferate and grow *in vitro* was recently begun with the demosponge *Suberites domuncula* (Müller & Brümmer, 1998; Custodio *et al.*, 1998; Müller *et al.*, 1999). One crucial step was the finding that single sponge cells, obtained by dissociation of sponge tissue, have lost their telomerase activity and hence their potency for an (unlimited) cell division (Koziol *et al.*, 1998). After formation of aggregates the cells regain telomerase activity and with this their growth potential; such cells also are able to differentiate in the aggregates, that were termed primmorphs (Müller & Brümmer, 1998; Custodio *et al.*, 1998; Müller *et al.*, 1999).

Biologically active compounds from *S. domuncula*

Neurotoxic compound

Already in 1906, it has been proposed that a bioactive/neurotoxic compound is present in *S. domuncula* (Richet, 1906). The term Suberitin was coined for this bioactive compound (Arndt, 1928) which was finally identified on protein level as a polypeptide which comprises lytic activity on cells (Cariello & Zanetti, 1979). In a recent approach, the extract of *S. domuncula* containing low molecular weight metabolites has been chemically analysed. A number of glycerol derivatives have been isolated and identified as lyso-PAF (platelet activating factor) congeners (to be published).

Quinolinic acid

In a controlled laboratory study partial apoptosis of sponge tissue was induced in *S. domuncula* by incubation with the Fe^{2+} chelator Dip for 5 days, or by deprivation of aeration (Schröder *et al.*, submitted). It has

been found that after induction of apoptosis, a mollusc (*Truncullariopsis trunculus*) is selectively grazing in certain affected areas of this sponge. A subsequent analysis of extracts from the apoptotic tissue revealed high neurotoxicity which could be abolished by (+)-MK-801/dizocilpine, a compound known to antagonize the agonistic effect of quinolinic acid on the NMDA receptor (Yoneda & Ogita, 1989). The presumably apoptosis inducing compound, quinolinic acid, was identified in a direct approach using high performance liquid chromatography (HPLC; Schröder *et al.*, submitted).

The primmorph system

In recent years, it was established that sponge cells can be cultivated *in vitro* as primmorphs (Custodio *et al.*, 1998; Müller *et al.*, 1999; Müller & Brümmer, 1998). Primmorphs are defined as a special form of *in vitro* cell culture which allows the formation of three-dimensionally organized aggregates that comprise proliferating and differentiating cells. We have focused on the formation of primmorphs from the demosponges *S. domuncula* (fig. 1A a), *Dysidea avara* (fig. 1A c) (Müller *et al.*, 2000) and occasionally *G. cydonium* (fig. 1A b).
The formation of primmorphs can technically be achieved as follows. Sponge tissue samples are transferred into CMFSW-E (Ca^{2+} and Mg^{2+}-free artificial seawater containing EDTA) and single cells are dissociated during shaking (fig. 1A d). After washing the cells are transferred into Ca^{2+} and Mg^{2+}-containing seawater, supplemented with antibiotics (100 IE/ml of penicillin and 100 µg/ml of streptomycin), and kept at 16°C. Immediately after transfer into the Ca^{2+} and Mg^{2+}-containing seawater and during shaking, the single cells form small, ~20 cells containing aggregates which grow in size during the subsequent three days to 1,000 µm large cell clumps (fig. 1A e). After usually five days primmorphs are formed (fig. 1A f). As the basal medium the natural seawater is enriched to 0.2% with RPMI1640-medium.

Characteristics

The primmorphs are marked by the presence of proliferating cells and a characteristic histology. The BrdU (5-bromo-2'-deoxy-uridine)-labelling and detection assay has been used to demonstrate that the cells organized in the primmorphs regain the capacity to proliferate. The percentage of BrdU-positive cells present in cell aggregates formed from single cells after one day in culture is low; only 6.5% of these cells are counted to be positive. In contrast, the number of DNA-synthesizing/proliferating cells present in primmorphs is high and reaches values of 20% to 30% depending on the age of the primmorphs.

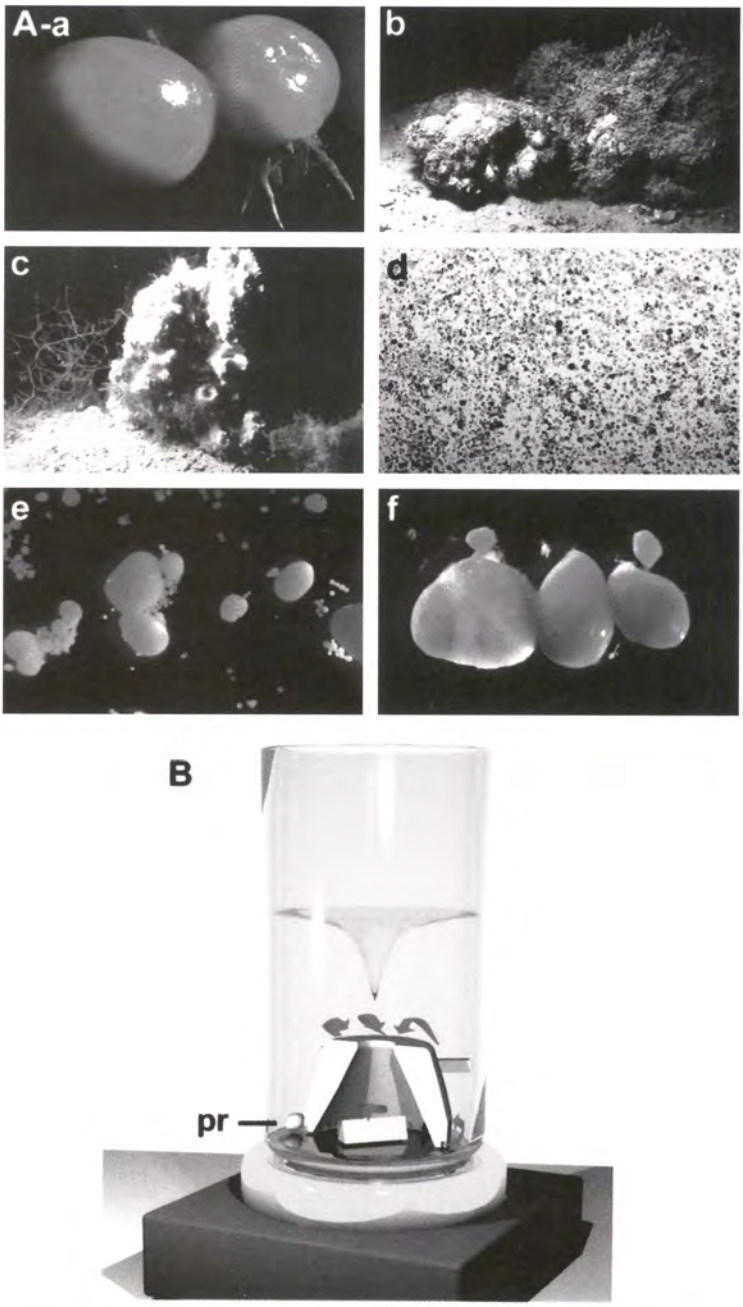

Figure 1 - Cultivation of primmorphs. (A) Primmorph formation from cells of the sponges *S. domuncula* (a) *G. cydonium* (b) and *D. avara* (c). Magnification; A x 0.8, B x 0.07 and C x 0.06. Primmorph formation from cells of *S. domuncula*: Dissociated single cells from *S. domuncula* [x 10] (d). After transfer to seawater aggregates are formed which reach a size of 1,000 µm after three days [x 10] (e). Primmorphs are formed after 5 days; [x 8] (f). (B) Bioreactor used for the production of secondary metabolites produced from sponge primmorphs. The current in the 1 liter beaker was driven by a magnetic stirrer which rotated in the cage. The water flow is indicated by the arrows and the primmorphs are marked (pr).

The diameter of the cell aggregates increases steadily after an incubation period of approximately three days. After a total incubation period of about five days, primmorphs are formed from cell aggregates. During the phase of primmorph formation the aggregates contract to round-shaped bodies, 1 to 5 mm in diameter, leaving behind detritus and dead cells. In the initial phase the primmorphs remain round-shaped but after incubation of longer than three to four weeks many of them adhere to the bottom of the culture dish. Cross sections through the primmorphs and microscopic analysis of sections stained with Ziehl's fuchsin revealed that the cells in the interior are surrounded by an almost complete single-cellular layer of epithelial-like cells. The cells which compose the squamous epithelium of the primmorphs are pinacocytes as judged from their flattened, fusiform extensions and their prominent nucleus. The cells inside the primmorphs are primarily spherulous cells while the rest may be termed amoebocytes and archaeocytes. The organized arrangement of the cells within the primmorphs distinguishes them from aggregates which are formed from dissociated cells in the presence of the homologous aggregation factor.

Medium design

The growth conditions could (until now) be optimized by supplementing the natural seawater/0.2% of RPMI1640-medium with silicate. Natural seawater contains < 5 µM silicate; however, the optimal concentration of silicate for cell proliferation and spicule formation is 60 µM (Krasko *et al.*, 2000, 2002). Furthermore, iron plays apparently an essential role, since it occurs usually in seawater at concentrations of < 1 nM; it was found, however, that the iron concentration, as Fe^{3+}, should be increased to 30 µM for optimal growth conditions of primmorphs (Le Pennec *et al.*, submitted). While the size of the primmorphs cultivated without additional silicate or Fe^{3+} is ≈ 2 mm, that of primmorphs grown in medium supplemented with 60 µM silicate reaches 6 mm. If seawater medium containing 60 µM silicate is further supplemented with 30 µM Fe^{3+} primmorphs reach a size of ≈ 10 mm (Le Pennec *et al.*, submitted).

Silicate and Fe^{3+} were found to display pronounced effects on gene expression in cells/primmorphs from *S. domuncula* as documented by the following experiments. Five days old primmorphs were incubated in seawater/RPMI1640-medium only or in this medium supplemented with 60 µM Na-silicate. The primmorphs were then incubated under these conditions for additional 5 days. During this period, the level of expression of the three genes, silicatein, collagen and myotrophin, was determined. Silicatein has previously been described as an enzyme which is primarily involved in the formation of sponge spicules (Cha *et al.*, 1999; Krasko *et al.*, 2000). Collagen is a major protein present in the extracellular matrix of sponges and functions also as a matrix for the spicule formation (Krasko *et al.*, 2000). Myotrophin is a proteinaceous growth factor from sponge (see below). The deduced aa sequence

of the *S. domuncula* silicatein is given in figure 2A to demonstrate its close sequence relationship to the catabolic enzyme cathepsin. However, unlike the protease sequence, silicatein does not comprise all three amino acids of the catalytic triad characteristic for cysteine proteases; in silicatein the residues His and Asn are found, while the third amino acid, Cys, is missing. Another difference in the silicatein sequence is a serine residue cluster which is lacking in cathepsin. The location of the three putative disulfide bonds, known from other cysteine proteases, is the same in both sequences. For the determination of the level of gene expression RNA was extracted from primmorphs after different periods of incubation in the absence or presence of 60 µM Na-silicate. Northern blotting experiments revealed that the expression of silicatein (fig. 2A a), collagen (fig. 2A b) and myotrophin (fig. 2A c) was low or even not detectable, if the primmorphs remained in medium not supplemented with silicate. However, if 60 µM of the Na-salt was added a strong up regulation of the expression of these three genes was seen.

A similarly strong effect on the level of gene expression was seen if primmorphs were incubated in medium optimized with 30 µM Fe^{3+} (fig. 2B). For this series of experiments the effect on the genes encoding ferritin, septin and a scavenger receptor was determined. Ferritin is an iron storage protein (reviewed in: Chasteen & Harrison, 1999); septin molecules are known to be involved in the completion of cytokinesis (reviewed in: Xie *et al.*, 1999), and hence can be used as marker proteins for cell division. Finally a protein was selected, the scavenger receptor, that might be involved in the growth guidance/control of the spicules formation. The genes encoding these three proteins were cloned from *S. domuncula* (Krasko *et al.*, 2002). The deduced polypeptide of the sponge ferritin molecule shows the characteristic two ferritin ion-binding regions (fig. 2B). Expression studies using these three molecular probes were performed to analyze their expression level in primmorphs after incubation in medium, supplemented with 30 µM Fe^{3+}. Only the expression of the septin gene was seen in primmorphs in the absence of Fe^{3+}, reflecting the proliferation of the cells in the primmorphs (fig. 2B b); after addition of Fe^{3+} the increase of the expression in primmorphs is striking even after 1 day. The expression of both ferritin (fig. 2B a) and the scavenger receptor (fig. 2B c) is only seen if the primmorphs were kept in medium enriched with 30 µM Fe^{3+}, underscoring the effect of this ion on sponge cell metabolism.

Other factors

One further growth promoting protein has been isolated from *S. domuncula* which was shown to stimulate proliferation of sponge cells; the myotrophin-like polypeptide (Schröder *et al.*, 2000). The cDNA of the sponge myotrophin was isolated; the potential open reading frame of 360 nt encodes a 120 aa long protein with a calculated M_r of 12,837.

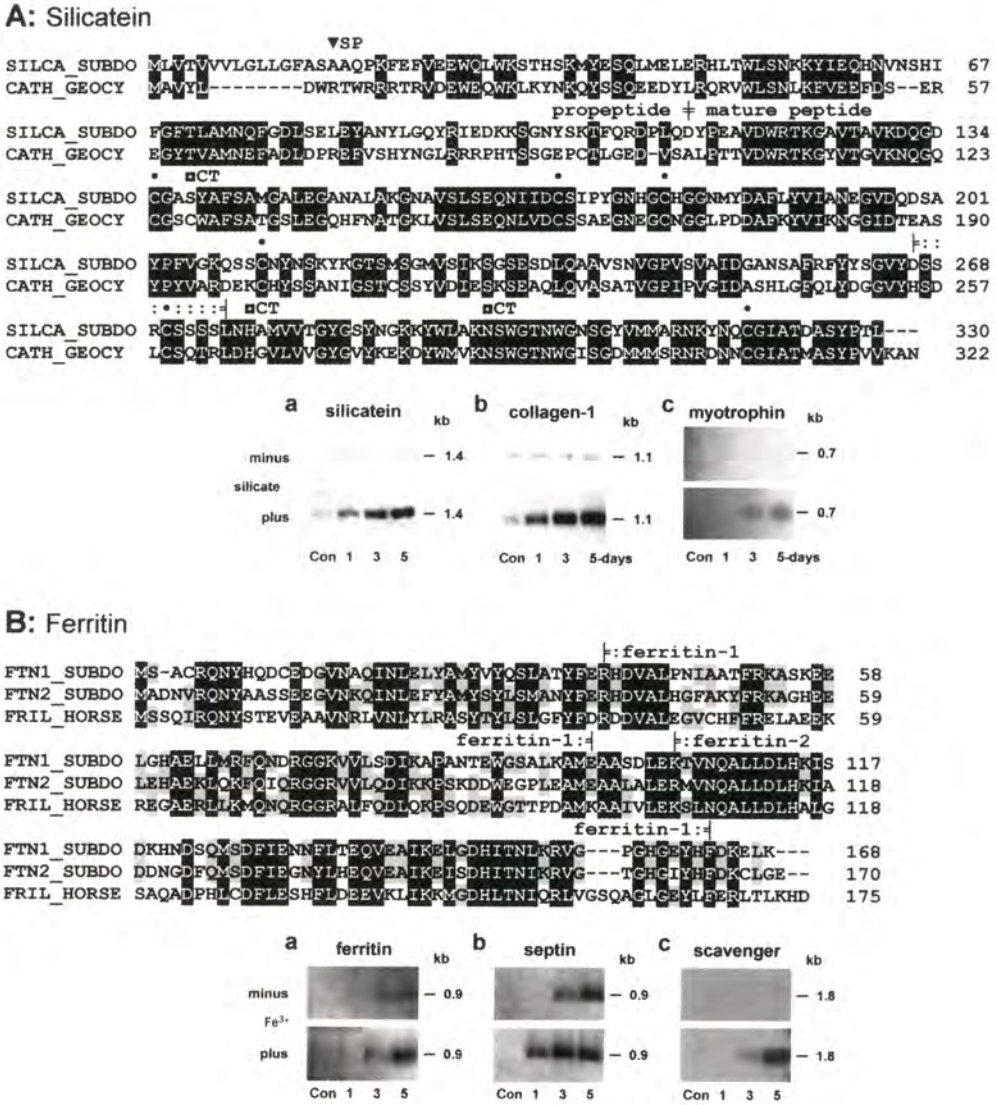

Figure 2 - Effect of silicate (A) and iron ions (B) on gene expression in primmorphs. (A) Sequence comparison: Silicatein polypeptide. The deduced aa sequence from the *S. domuncula* cDNA, *SDSILICA*, termed SILICA_SUBDO, is aligned with the cathepsin L-like protease precursor from the sponges *G. cydonium* (CATH_GEOCY; Y10527). Residues conserved (similar or related with respect to their similar physico-chemical properties) in both sequences are shown in white on black. The characteristic sites within the silicatein sequences are indicated: the three aa Cys, His and Asn form the catalytic triad (◘,CT), the 17-residues long signal peptide (▼; SP), the processing site for the conversion of the proenzyme to the mature enzyme, the cysteine residues potentially involved in the three putative disulfide bonds (•), as well as the serine cluster (╞:::╡). Northern blotting: 5 days old primmorphs were incubated for 0 (controls; Con) to 5 days either in the absence of exogenous silicate (minus silicate) or presence of 60 µM Na-silicate (plus silicate). RNA was extracted and 5 µg of total RNA was size separated; after blot transfer hybridization was performed either with the *silicatein* probe (a), the *collagen* probe (b), or the *myotrophin* probe (c), all isolated from *S. domuncula*. (B) Sequence comparison: Ferritin polypeptide. The deduced aa sequences from the *S. domuncula* cDNA, *SDFTN1* (FTN1_SUBDO; AJ306615), and *SDFTN2* (FTN2_SUBDO; AJ306615), are aligned with the corresponding sequence from the horse L-ferritin (FRIL_HORSE, P02791). The characteristic two ferritin ion-binding region signatures (╞:ferritin-1:╡ and ╞:ferritin-2:╡) are indicated. Northern blotting. Primmorphs were incubated for 5 days in seawater, supplemented with 60 µM silicate. Then the primmorphs were transferred into seawater with silicate which had been complemented with 30 µM Fe^{3+}; the primmorphs were incubated for 0 (controls; Con) up to 5 days in the absence (minus Fe^{3+}; open bars) or presence of 30 µM Fe (plus Fe^{3+}; closed bars). The expression of *ferritin* (a), *septin* (b) and *scavenger receptor* (c) was determined. Northern blotting was performed with the labelled probes and with 5 µg of total RNA which were loaded on the slots.

The sequence shares high similarity with the known metazoan myotrophin sequences. Recombinant sponge myotrophin was prepared and found to stimulate overall protein synthesis by 5-fold (Schröder *et al.*, 2000).

Besides the chemical factors mentioned above physical factors were found to be essential for a further growth of the primmorphs followed by a reconstruction of a specimen. For example, the water current is critically important for the survival of primmorphs and their growth. Therefore a bioreactor was developed which allows the cultivation of primmorphs at stronger current (Le Pennec *et al.*, submitted; fig. 1B). Incubation of primmorphs under such conditions resulted in the formation of canals in the primmorphs and the expression of the homeobox gene *Iroquois*.

Production of bioactive compounds in the primmorph system

In a first approach to scale up the production of bioactive compounds in the primmorph system, the primmorphs were incubated in a small bioreactor of a volume of 1 liter (fig. 1B). The current is generated by magnetic stirrer within a cylinder that sucks in the water from the top and extrudes it through the holes in the bottom into the external space where the primmorphs are kept.

Avarol

The first successful production of a sponge secondary metabolite in a bioreactor was achieved with primmorphs from *D. avara* that produced avarol (Müller *et al.*, 2000). Earlier studies revealed that avarol has potent antiviral (Sarin *et al.*, 1987) and antitumor activity (Müller *et al.*, 1985 a,b) as well as anti-inflammatory activity (Ferrándiz *et al.*, 1994). Cells from *D. avara* were prepared as described (Müller *et al.*, 2000). They were transferred into a bioreactor and incubated either in Ca^{2+}- and Mg^{2+}-free seawater/0.2% of RPMI1640-medium, which allows no re-association into aggregates and further into primmorphs. Or, the cells were incubated in a bioreactor using Ca^{2+}- and Mg^{2+}-containing seawater/0.2% of RPMI1640-medium which promotes the formation of primmorphs from single cells after ≈3 days (Müller *et al.*, 2000). Avarol was extracted with ethyl acetate (EtOAc) and subsequently identified and quantified by high-performance liquid chromatography (HPLC; Müller *et al.*, 2000). The analysis revealed that sponge single cells contained only minute amounts of the secondary metabolite (fig. 3, single cells), while in the primmorphs considerable amounts of avarol could be demonstrated (fig. 3, Primmorphs). In the absence of Fe^{3+} as well as of silicate the primmorphs contained ≈1 µg avarol/100 µg protein after a 6- or 12 days incubation. The production of avarol could be enhanced by addition of Fe^{3+} to the incubation medium. Supplementing the seawater/0.2% of RPMI1640–medium with 30 µM Fe^{3+} increased the level of avarol in the primmorphs to 1.5 and 2.5 µg/100 µg of protein in 6 days or 12 days old primmorphs, respectively. In con-

trast, a supplementation of the medium with silicate did not significantly change the avarol level when compared to the control. It is interesting to note that the primmorphs cultivated in the presence of Fe^{3+} are able to synthesize as much avarol as cells from animals taken from the field (1.8 µg avarol/100 µg protein; fig. 3).

These data indicate that the primmorph system is useful for an *in vitro* production of sponge secondary metabolites and underline again that single cells from sponges, at least to the present state of knowledge, are not capable to produce secondary metabolites and are, as shown earlier (Custodio *et al.*, 1998; Müller *et al.*, 1999) not able to proliferate.

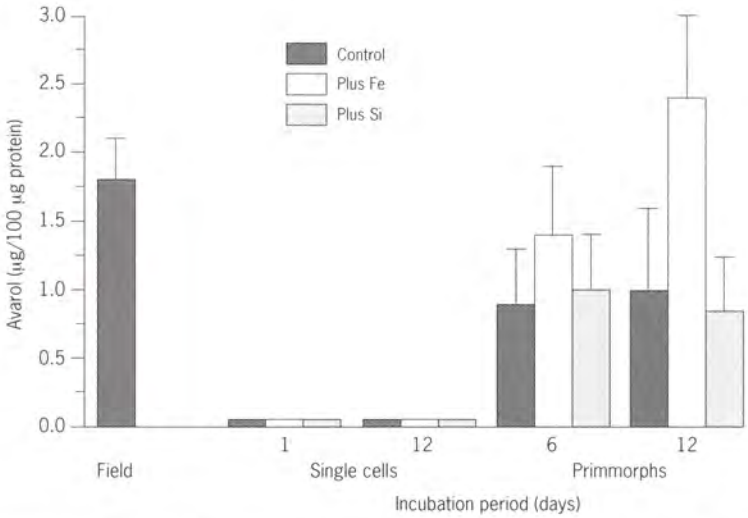

Figure 3 - Production of the secondary metabolite avarol in a bioreactor using primmorphs from *D. avara*. The dissociated cells were either kept in Ca^{2+}- and Mg^{2+}-free seawater/0.2% of RPMI1640–medium; under these conditions the cells remained in the single state. In parallel, primmorphs were prepared from single cells by incubation in seawater[Ca^{2+}- and Mg^{2+}-containing]/0.2% of RPMI1640–medium. For the extraction of avarol, cultures containing either single cells (incubation in Ca^{2+}- and Mg^{2+}-free medium) or primmorphs (incubation in Ca^{2+}- and Mg^{2+}-containing medium) were used (black bars). In separate series of experiment, those media were supplemented with either 30 µM of Fe^{3+} (ferric citrate; open bars) or 60 µM of silicate (Na-hexafluorosilicate; crosshatched bars). At the indicated incubation period samples were extracted with EtOAc and the extract was separated by HPLC. In the fractions containing avarol, its concentration was determined and the values are given as µg /100 µg of protein. Five experiments were performed each; the means ± SD are given. The values of avarol in cells/primmorphs are correlated with the level of this secondary metabolite found in specimens from the field.

(2'-5')oligoadenylates

The (2'-5')oligoadenylate synthetase [(2-5)A synthetase] system (Rebouillat *et al.*, 1999) is one known efficient protection against invading viruses/microorganisms. The (2-5)A synthetase(s) is(are) activated by certain classes of RNA; furthermore, the (2-5)A pathway in vertebrates is induced by interferons (Pestka *et al.*, 1987). The major enzyme of

this pathway, the (2-5)A synthetase catalyzes the synthesis of a series of 2'-5'-linked oligoadenylates from ATP, termed (2-5)A [= pppA(2'p5'A)$_n$ [p_nA_n], with chain lengths of 1 £ n £ 30] (Hovanessian, 1991). Surprisingly, the (2-5)A synthetase (Wiens *et al.*, 1999) as well as its reaction products (Kuusksalu *et al.*, 1995, 1998) have been identified in sponges (first in *G. cydonium*) while this system is absent in protostomian animals, e.g. in *Drosophila melanogaster* or *Caenorhabditis elegans*. Recently, we succeeded to demonstrate that primmorphs from *S. domuncula* also produce (2-5)A (Grebenjuk *et al.*, 2002). Primmorphs were prepared and used for the analysis of (2-5)A after a total incubation period of 5 days. As summarized in figure 4, the level of (2-5)A in primmorphs was ≈1.5% (controls) with respect to the concentration of the ATP substrate in the assay (incubation period of 24 hrs). A significant stimulation of the (2-5)A production could be observed after incubation of primmorphs with lipopolysaccharide (LPS). LPS is an endotoxin derived from the outer cell wall of gram-negative bacteria which binds to the cell surface molecule CD14 (Ulevitch & Tobias, 1994). If primmorphs were incubated with 1 µg/ml of LPS the level of (2-5)A production increased to 3.3% of the ATP substrate applied to the assay (fig. 4); less pronounced was the rise if the amount of LPS was increased to 10 µg/ml. The amount of (2-5)A in control primmorphs is as high as that measured in tissue from an animal which had been kept for six months in the aquarium (1.12% of the ATP converted to (2-5)A).

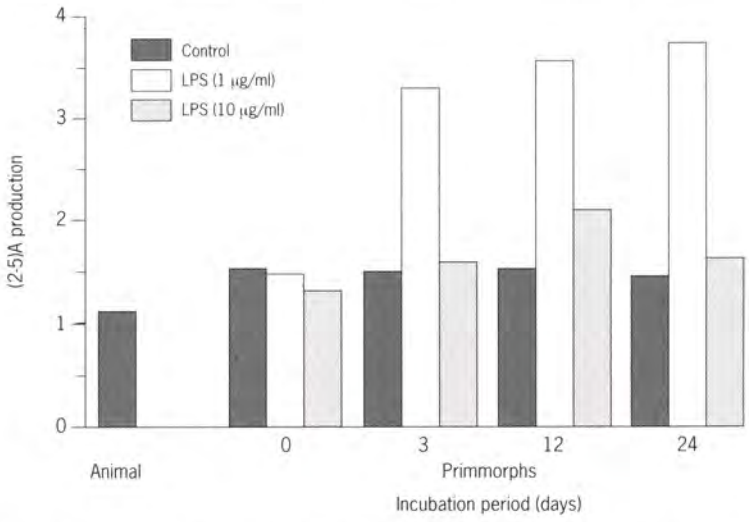

Figure 4 - Production of (2-5)A in primmorphs from *S. domuncula*. Five days old primmorphs were incubated in seawater/0.2% with RPMI1640-medium and incubated in the absence of LPS (control; filled bars), or presence (1 [open bars] or 10 µg/ml [crosshatched bars]) of LPS for 0 to 24 hrs. The products were dephosphorylated and analyzed by HPLC. The reaction products were also analyzed by TLC, followed by autoradiography to determine the product. Based on these data the conversion of [14C]ATP to (2-5)A was calculated and is given in percentage to the sum of ATP, ADP and AMP. In parallel, the level of (2-5)A in an animal kept for six months in the aquarium was determined.

Future directions

The techniques available to utilize sponge cells as source for the isolation of secondary metabolites are restricted to primmorphs. Using this system it could be established that primmorphs from *D. avara* retain the capacity to synthesize avarol, the major pharmacologically active compound of this sponge, and primmorphs from *S. domuncula* to synthesize the immunomodulator (2-5)A. It might be stressed here that the efficiency of the primmorph system can be modulated by addition of defined components to the culture medium, e.g. iron [Fe^{3+}]. It has been shown that after dissociation of sponge tissue into single cells, aggregates are formed in the presence of Ca^{2+} ions which allow the cells to proliferate and-in parallel with this process-to differentiate from archaeocytes to epithelial-like cells. During this course primmorphs are formed. It is important to mention that in those cells the expression of growth controlling genes (e.g. myotrophin or septin) and of genes involved in morphogenetic events (e.g. silicatein or ferritin) is upregulated. These genes trigger the cells of the primmorphs either only in the *proliferation phase* or also in the *morphogenesis phase*. Two major controlling extracellular factors have been identified which differentially modulate the onset of these phases: iron ions and silicate. While iron is required for the increased production of the secondary metabolite avarol, as shown above, iron together with silicate pushes the cells in primmorphs into the morphogenesis phase. The latter effect could be demonstrated by the observation that primmorphs, cultured in the presence of iron and silicate, form the major skeletal elements of the demosponges, spicules and the extracellular matrix molecule collagen (Krasko *et al.*, 2000, 2002; Le Pennec *et al.*, submitted). One consequence of this process is the formation of the canals in primmorphs which is paralleled with the expression of one gene, encoding a *Iroquois* homeobox protein (to be published). *Iroquois* genes are expressed in mammalian systems during vascular differentiation of cells (Olson & Srivastava, 1996).

The major challenge for our future work in this direction is the identification of additional factors which allow the optimization of the growth conditions for the primmorphs to remain in the proliferation state. During proliferation the primmorphs appear to be optimal to be used for bioprocessing technologies. It is expected that both proteinaceous and non-proteinaceous molecules [especially isoprenoids], as well as inorganic components will be identified which allow the primmorphs to enter the morphogenesis phase. We are optimistic to reach this goal, especially in view of our present attempts to analyze the expressed genome of *S. domuncula* (*http://spongebase.uni-mainz.de/cgi-bin/blast/blastserver.cgi*).

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The influence of silicate on *Suberites domuncula* primmorphs

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Abstract

Primmorphs of the marine sponge *Suberites domuncula* were maintained in seawater at different silicate concentrations. We observed that in the presence of high silicate concentrations (70 and 148 µM) attachment of primmorphs to a solid matrix was stimulated, while at low concentrations (3.5 and 25 µM) attachment of primmorphs was never observed. In addition, we observed that spicule formation had occurred in primmorphs in the presence of high silicate concentrations.

Introduction

Since Bergmann & Feeney (1950, 1951) isolated bioactive compounds from sponges in the early fifties, many interesting natural products have been discovered in sponges. However, commercial applications have been hampered by the lack of a suitable production system. Wild harvest, aquaculture and cell culture have not yet resulted in a solution for the supply problem (Osinga *et al.*, 1999). Some of these problems may be overcome by culturing sponges *in vitro* as dense sphere-shaped axenic aggregates of 1-2 mm in size, which are termed primmorphs. Primmorphs can be maintained in unsupplemented seawater for more than 5 months (Custodio *et al.*, 1998) and it was shown that sponge cells in primmorphs retain their telomerase activity, which is the genetic indicator for proliferative capacity, while dissociated sponge cells rapidly lose their telomerase activity (Koziol *et al.*, 1998; Custodio *et al.*, 1998; Müller *et al.*, 1999). The sponge skeleton is important for the internal organisation and development of a growing sponge (Bergquist, 1978). Thus if a primmorph is able to develop into a functional sponge it probably needs silicate, the building block of the spicule skeleton of demosponges. Krasko *et al.* (2000) showed that silicate induced the formation of silicatein, an enzyme which catalyses deposition of inorganic silica under the formation of spicules. They observed that in addition to the formation of silicatein, silicate induces production of myotrophin. Myotrophin from *Suberites domuncula* has the potency to stimulate the production of collagen, an important skeletal element in sponges, in the primmorph system (Schröder *et al.*, 2000). It is assumed that myotrophin

is released by sclerocytes, motile secretory cells which produce spicules, and causes the expression of genes involved in the production of collagen-like spongin in exopinacocytes.
Our objective is to study if silicate triggers the further development of primmorphs to develop into functional sponges.

Material and methods

Material

Sponge

Specimens of *Suberites domuncula* that were used for our experiments originated from gemmules that were collected near Rovinj (Croatia) and developed into functional sponges in our aquarium at a temperature of approximately 17°C.

Solutions

Artificial seawater (ASW) was prepared from 33 g/l instant ocean reef crystals (Aquarium Systems, Sarrebourg, France) in demineralised water and supplemented with 1% penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml) solution (Life Technologies, Paisley, UK). The pH was adjusted to 8.2 with HCl and the solution was filter sterilised (pore size 0.22 µm; Nalgene, Rochester, USA). ASW containing silicate was prepared by addition of sodium metasilicate (Sigma, St. Louis, USA) to ASW before filtration.
Natural seawater (NSW) was purchased from Sigma and supplemented with 1% penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml) solution (Life Technologies).
Calcium- and magnesium-free seawater (CMFSW) was prepared by dissolving 0.994 g Na_2SO_4, 0.0168 g $NaHCO_3$, 0.746 g KCl, 31.6 g NaCl and 2.42 g Tris in 1 l demineralised water. CMFSW was supplemented with 1% penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml) solution and pH was adjusted to 8.2 before filter sterilisation (pore size 0.22 µm).
A sterile 90% Percoll stock solution was prepared by adding a sterile 10-fold concentrated solution of CMFSW to 100% Percoll (Sigma). The stock solution was diluted with sterile CMFSW to obtain 10% and 60% Percoll solutions.

Experimental

Preparation of a sponge-cell suspension and maintaining primmorphs

The protocol used to prepare a sponge-cell suspension was based on the method of Custodio *et al.* (1998). Approximately 1 ml sponge tissue was submersed in artificial seawater (ASW) and cut into pieces of 1 mm^3. The pieces were washed once more in ASW and transferred into a 50 ml conical tube (Greiner, Solingen, Germany) containing 25 ml of calcium- and magnesium-free seawater (CMFSW). The tube was placed horizontally in a shaking incubator (type GFL, Salm en

Kipp, Breukelen, the Netherlands) at low speed and at a temperature of 16-20°C. After 60 min the supernatant was discarded and replaced by 25 ml of CMFSW and the tube was returned into the shaking incubator. After 120 min the supernatant was filtered through a sterile 50 µm mesh nylon (supernatant 1) and replaced by 25 ml of CMFSW. The tube containing the sponge pieces was incubated for 120 more min before supernatant 2 was harvested by filtering the cell suspension through a 50 µm mesh nylon. Supernatant 1 and 2 were centrifuged for 10 min at 800 x g and the pellets were suspended and pooled in 5 ml CMFSW.

Ten millimeters of a 60% Percoll solution were pipetted in a sterile conical tube (Greiner) and 10 ml of a 10% Percoll solution were pipetted on top of the 60% layer. The cell suspension in CMFSW was pipetted carefully on top of the 10% layer and the tube was centrifuged at 1000 rpm (138 x g) at 4°C for 10 minutes. A sterile Pasteur pipette was used to remove the middle layer containing the sponge cells and to transfer it into a sterile tube. A large volume of CMFSW (40-50 ml) was added to the cell suspension and it was centrifuged at 800 x g for 10 minutes. Subsequently the pellet was resuspended in 5 ml of CMFSW. The sponge-cell concentration was determined by using a Neubauer Improved haemocytometer and an inverted light microscope (Olympus CK2, Olympus, Hamburg, Germany). Subsequently, the cell suspension was centrifuged for 10 min at 800 g and the pellet was dissolved in ASW to establish a cell concentration of 2.106 cells/ml. Aliquots of 6 ml cell suspension were transferred to polyethylene 60 mm Petri dishes (Greiner). The Petri dishes were incubated under gentle agitation on a rocking plate. After 7 days, individual primmorphs with a diameter of at least 1 mm were picked from the Petri dish with a sterile spatula and transferred to separate wells of a 24-well plate (Corning, New York, USA). Each well contained 1.5 ml ASW, which was refreshed every week.

Effect of silicate

To study the effect of silicate on primmorphs, 24 primmorphs were maintained in seawater containing different silicate concentrations (3.5 µM in NSW and 25 µM, 70 µM and 148 µM in ASW) in separate wells of a 24-well plate. Primmorphs from two *Suberites domuncula* specimens were used for the experiment. Primmorphs from one sponge were used to test silicate concentrations of 3.5 and 148 µM, while primmorphs from another specimen were used to test silicate concentrations of 25 and 70 µM. During the experiment the primmorphs were inspected regularly with an inverted light microscope (Olympus CK2) and a stereomicroscope (SV11, Zeiss, Jena, Germany). The silicate concentrations that were applied were checked by spectrophotometric analysis. The protocol was adapted from Strickland and Parsons (1972). Only plastic materials were used to prevent release of silicate from glassware. A calibration curve was made from 0 to 100 µM. For each sample or standard, 0.25 ml molybdate solution (13.33 g $(NH_4)_6Mo_7O_{24}.4H_2O$ per

liter double distilled water (dds) was pipetted into a tube and subsequently, 0.625 ml sample or standard was added. The samples were mixed and after 10 min, 0.375 ml reduction reagent was added (100 ml reduction reagent: 40 ml ascorbic acid solution (28 g ascorbic acid/l dds); 20 ml oxalic acid (saturated solution in dds); 16.7 ml sulphuric acid (90%); 23.3 ml dds). The samples and standards were incubated in a 50°C water bath for 30 minutes. Subsequently, the extinction of the standards and samples was measured with a spectrophotometer (Spectronic-20 Genesys, Spectronic Instruments, Rochester, USA) at a wavelength of 810 nm. A standard containing 0 µM silicate was used to calibrate the zero value of the spectrophotometer.

Results and discussion

The influence of silicate on primmorphs was tested by exposing primmorphs to different silicate concentrations. The primmorphs that were maintained at silicate concentrations of 3.5 and 25 µM showed no growth or morphological changes. After two days, fungi were growing on the surface of five of the primmorphs in seawater containing 3.5 µM silicate. The fungal biomass formed a web surrounding the primmorphs, that remained intact. Within 14 days these primmorphs had turned from orange into black and they were discarded. Some of the primmorphs in ASW containing 25 µM silicate started disintegrating 2 weeks after starting the experiment. The smooth skin had disappeared and morphology had changed in such a way that the primmorphs resembled early-stage primmorphs and some spicules could be observed. Some released cell aggregates from the disintegrating primmorphs were attached to the well, but the rough primmorphs itself did not attach. After 55 days all 6 primmorphs were disintegrated and they were discarded.

Within 14 days after the addition of 70 µM silicate to 6 primmorphs, no changes in outer appearance occurred. However, in the period between 14 and 28 days after the addition of silicate 3 out of the 6 primmorphs attached to the bottom of the well. The spherical shape and the smooth skin of the primmorph disappeared and a more or less 2-dimensional tissue-like attached structure was formed in which the presence of spicules was observed (fig. 1A). The number of spicules in the tissue seemed to increase during time. They were not organised in the sponge tissue, but distributed randomly, which is also the case in tissue of mature *Suberites domuncula*.

The spicules that were present in the sponge tissue must have been produced by the primmorph and the tissue-like aggregate during the experiment. They cannot originate from the parent sponge that was used to prepare primmorphs, because microscopic analysis had revealed that after gradient centrifugation all spicules from the parent sponge had been removed from the sponge-cell suspension. During the period from 4 to 6 weeks, no changes were observed for the attached sponge tissue-like

aggregates in the wells. However, after 6 weeks the size of the tissue-like aggregate started decreasing, while spicules that were present in the exterior parts of the tissue were left behind (fig. 1B). This was probably caused by a deficiency of nutrients to maintain the sponge tissue. In the period between 6 and 8 weeks after starting the experiment the 3 smooth primmorphs that had not attached started to disintegrate. This occurred 3 months after the preparation of the primmorphs and might be the result of age of the primmorphs.

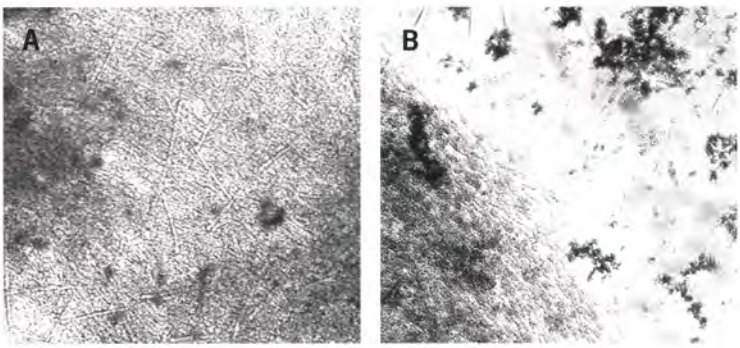

Figure 1
(A) Part of the tissue of an attached *Suberites domuncula* primmorph containing many spicules, magnification 200x.
(B) Spicules that were present in the sponge tissue were left behind when the sponge tissue decreased in size, magnification 100x.

The primmorphs that were incubated in ASW containing 148 µM silicate originated from the same sponge as the primmorphs that were maintained at a silicate concentration of 3.5 µM. Also these primmorphs at 148 µM silicate were covered with a fungal layer within 2 days. However, attachment of primmorphs in the presence of a high silicate concentration was not disturbed. After 6 days 1 of the 6 primmorphs attached to the bottom of the well and after 8 days 3 primmorphs were attached. Some spicules could be observed, but it was difficult to look into the dense biomass clump. No spreading of the cell mass was observed as in the case with 70 µM silicate. This might be caused by the presence of fungal biomass, which increased clearly during time. The colour of the primmorph changed from orange to black within 14 days, which indicates that the sponge cells were dying.

Although we have not done a quantitative analysis of the number of spicules at different silicate concentrations, it was obvious that more spicules were formed in primmorphs that were incubated in ASW containing 70 µM silicate than at 25 µM silicate. The observation that the number of spicules increased after attachment of the primmorph implies that sclerocytes were active in the attached sponge tissue. It is difficult to compare spicule formation in primmorphs at low and high silicate concentrations due to differences in development and the presence of fungi at the concentrations of 3.5 and 148 µM silicate. However, these results confirm the general idea that a positive correlation exists between silicate concentration, silicate uptake rate and spiculogenesis (Frøhlich & Barthel, 1997; Reincke & Barthel, 1997; Mercurio *et al.*, 2000).

The enhanced spiculogenesis is explained by the finding that the formation of silicatein increases strongly at concentrations of 60 µM silicate when compared to the formation in the presence of 1 µM silicate (Krasko *et al.*, 2000).

Silicate has also been found to stimulate the biosynthesis of myotrophin, which enhances the production of collagen in *Suberites domuncula* cells *in vitro* (Schröder *et al.*, 2000). It is assumed that myotrophin is released by sclerocytes and causes the formation of collagen-like spongin in exopinacocytes. At a concentration of 60 µM silicate the production of collagen was found to be approximately 700% higher than in seawater containing 1 µM silicate (Krasko *et al.*, 2000). A difference in collagen production in primmorphs in seawater containing a low silicate concentration (3.5 and 25 µM) in comparison to primmorphs in seawater containing a high silicate concentration (70 and 148 µM) is probably the explanation why only primmorphs in seawater containing a high silicate concentration attached to the substratum. It is well-known that collagen plays an important role in both the attachment of gemmules to a substratum and their subsequent morphogenesis (Mizoguchi & Watanabe, 1990). The basopinacoderm, which is the first layer of cells to attach to a substratum contains many collagen fibrils (Garonne, 1984). Inhibitors of collagen synthesis such as azetidine-2-carboxylic acid and α,α^1-dipyridyl prevent spreading of the basopinacoderm. At a concentration of 1.0 mM of azetidine-2-carboxylic acid gemmules did not attach to a substratum (Mizoguchi & Watanabe, 1990). Shimizu & Yoshizato (1993) discovered that inhibitors of collagen biosynthesis did not affect the reaggregation of dissociated sponge cells, but caused incomplete morphogenesis in the processes of spreading and development. These data combined with our results indicate that the exogenous silicate concentration is a trigger for both attachment and further development of primmorphs (fig. 2).

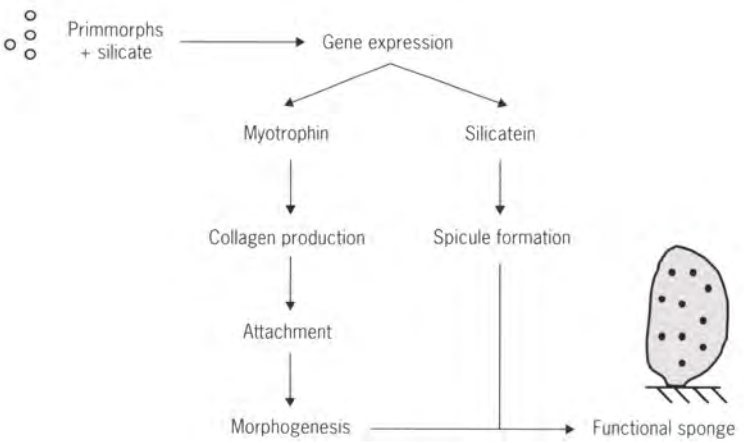

Figure 2
Postulated formation of a functional sponge triggered by silicate. Modified scheme from Krasko *et al.*, 2000.

Conclusion

Attachment of primmorphs and development of spicules are induced by the presence of high silicate concentrations (> 70 µM) in seawater. This might be the first step in the development of a functional sponge from a primmorph.

Acknowledgements
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Bioactive potential of bacteria associated with the marine sponge *Suberites domuncula*

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Abstract

The bacteria associated with marine invertebrates are found to be a rich source of bioactive metabolites. In the present study, the bacteria associated with sponge *Suberites domuncula* and its primmorphs (three dimensional aggregates containing proliferating cells) were isolated and cultured. These bacteria were extracted and the extracts were assayed for haemolytic, antimicrobial and cytotoxic activities. The bacteria having bioactive potential were phylogenetically identified following sequencing of the 16S rRNA gene. Extracts obtained from all the five bacterial strains isolated from sponge and its primmorphs showed haemolytic activity. The sponge associated bacteria belonging to alpha subdivision of Proteobacteria and the primmorph associated bacterium which was identified as a possibly novel *Pseudomonas* sp. displayed remarkable antimicrobial activities. An important observation of this study was that these bacterial extracts were active against multi-drug resistant clinical isolates such as *Staphylococcus aureus* and *Staphylococcus epidermidis* strains isolated from hospital patients. The bacterial extracts showing antimicrobial activity also showed cytotoxicity against HeLa and PC12 cells. In summary, this investigation explores the importance of sponge associated bacteria as a valuable resource for the discovery of novel bioactive molecules.

Introduction

In recent years, the ocean has been considered as a rich source of bioactive compounds possessing novel structures and biological activities. Marine bacteria alone constitute 10% of the total living biomass carbon of the biosphere. However, only a small percentage of marine bacteria have been studied for their bioactive potential. These bacteria originate mainly in sediments but also occur in the open ocean and associated with marine organisms. Sponges harbour large amounts of

bacteria that can amount to 40% of the biomass of the animal (Vacelet, 1975) which may exceed the bacterial concentration of the seawater by two orders of magnitude (Friedrich *et al.*, 2001). These bacteria can be explored for their potential to produce bioactive metabolites.
In the present investigation, the marine sponge *Suberites domuncula* (Demospongiae, Hadromerida, Suberitidae) was chosen as a source for the recovery of bacteria, having bioactive potential (fig. 1).

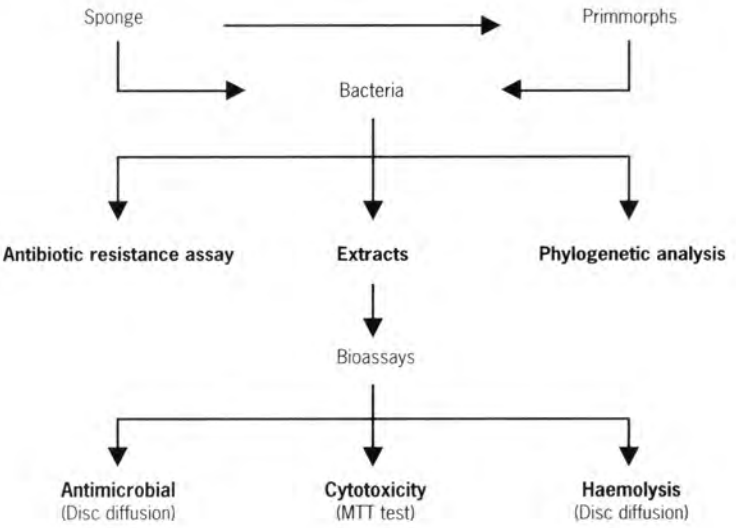

Figure 1
Schematic outline of experimental protocol.

Material and methods

Sponge collection

Suberites domuncula sponges were collected from depths of between 30-40 m, from the Northern Adriatic, near Rovinj, Croatia (45°07' N; 13°39' E) and kept in recirculating seawater aquaria in Mainz (Germany) under continuous aeration (Müller *et al.*, 1999).

Sponge primmorphs culture

Primmorphs were obtained from dissociated cells of *S. domuncula* as described previously (Müller *et al.*, 1999). Seven days after transfer of the cells into seawater, primmorphs were used for the isolation of bacteria.

Isolation of bacteria and preparation of bacterial extracts

A method described by Wahl *et al.* (1994) was adopted to isolate the sponge surface-associated bacteria. Isolated bacteria were cultured on B1 medium. (0.25% peptone, 0.15% yeast extract, 0.15% glycerol, 1.6% agar, 100% seawater) (Newbold *et al.*, 1999). Bacteria were isolated from

seven days old primmorphs of 3 to 5 mm in diameter and cultured on B1 medium. Bacterial extracts were prepared in n-butanol by using the method of Elyakov *et al.* (1996). Dry residues were stored below 5°C until further use.

Antimicrobial and haemolytic assays

Antibacterial, antifungal and haemolytic activities were tested using the standard paper disc diffusion method against gram-positive and gram-negative bacteria, yeast, and upon application on sheep blood agar (Oxoid). The diameter of the inhibition zones (diameter of inhibition zone minus diameter of disc) was measured in millimetre after incubation at 30°C for 24 hours.

Cytotoxicity assays

Bacterial extracts were tested for cytotoxicity against HeLa and PC12 cells. The cells were seeded into 96-well plates at a concentration of 1.4×10^4 cells cm^{-2} and incubated at 37°C. After 24 h incubation, bacterial extracts at different concentrations were added to the wells containing cells. The final volume in each well was 200 µl. The plates were then incubated at 37°C for 72 hours. Cell viability was determined using the MTT colorimetric assay (Scudiero *et al.*, 1988).

Antibiotic resistance assay

All the five bacterial strains were studied for their resistance against the standard antibiotics penicillin and streptomycin. The assay was carried out using the well-diffusion method.

Phylogenetic identification

PCR amplification, cloning, sequencing and phylogenetic analysis of the bacterial isolates from *S. domuncula* and its primmorphs was carried out as described previously (Hentschel *et al.*, 2001). The obtained sequences were aligned using the ABI prism auto assembler v. 2.1 software (Perkin Elmer, Foster City, CA, USA) and entered into the BLAST and ARB 16S rDNA sequence database (*www.arb-home.de*).

Results and discussion

The present study was an attempt to investigate the bioactive potential of sponge surface-associated bacteria which are thought to involve in the epibacterial chemical defence of the sponge *S. domuncula* (fig. 2) (Thakur *et al.*, submitted). Subsequently, biological activities of primmorph-associated bacteria were also studied as these bacteria are reported to have an antifouling activity (Thakur *et al.*, submitted).
Altogether six bioactive bacterial strains (SB1-SB6) were isolated from the sponge surface, whereas two bacterial strains (PB1-PB2) were isolated from its primmorphs.

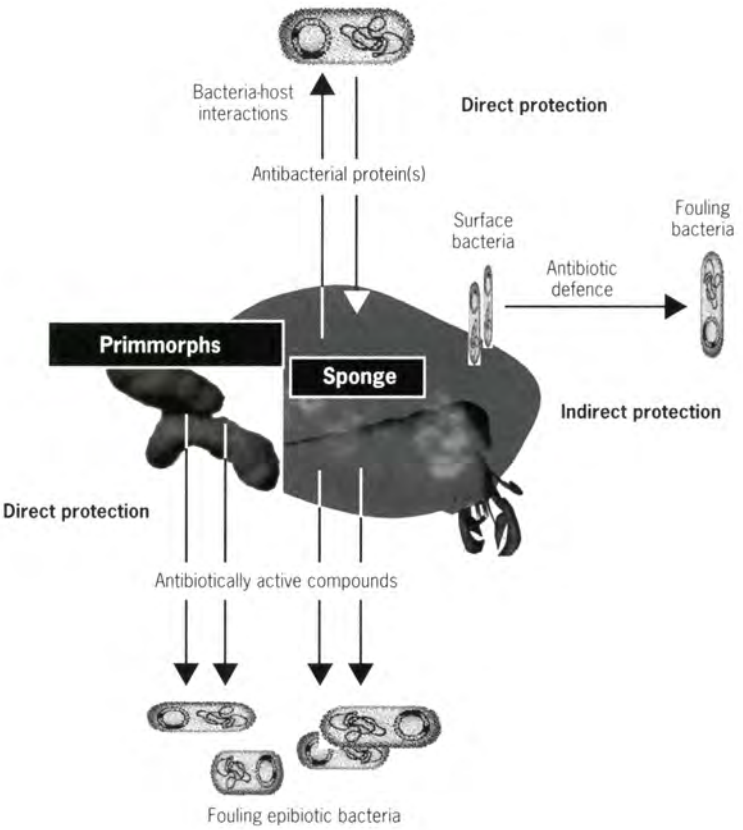

Figure 2
Schematic diagram of the direct and indirect epibacterial defence hypothesis for the sponge *S. domuncula*.

Bioactivity assays

The extracts obtained from sponge surface bacteria SB1, SB2 and primmorph-associated bacterium PB2 displayed antimicrobial activity (tab. 1). An important observation of this study was that these bacterial extracts were active against multi-drug resistant clinical isolates such as *Staphylococcus aureus* and *Staphylococcus epidermidis* strains isolated from hospital patients. These two bacteria are the most common hospital acquired infections and account for complications following surgical procedures (Smith & Jarvis, 1999; Raad *et al.*, 1998; De Lalla, 1999). All bacterial extracts also showed haemolytic activity. PC12 and HeLa cells are widely used in toxicity assays. In this study, we found that the extract obtained from sponge surface bacterium SB2 was toxic against PC12 cells (fig. 3). It was interesting to note that the extracts from both the primmorph associated bacteria PB1 and PB2 exhibited potent cytotoxicity against HeLa cells (fig. 3).

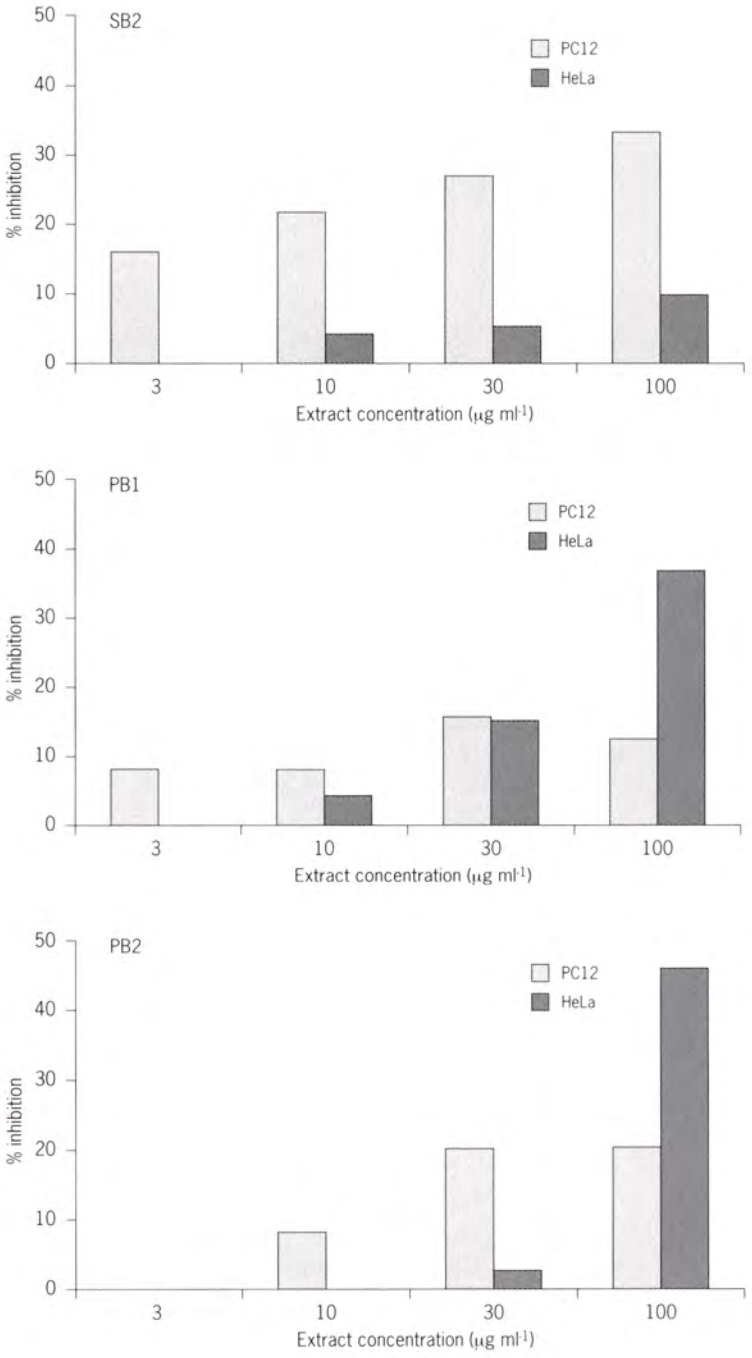

Figure 3
Cytotoxicity of SB2, PB1 and PB2 extracts against PC12 and HeLa cells.

Table 1 - Antimicrobial activity of bacterial extracts.

Test bacterial strains	SB1	SB2	SB6	PB1	PB2
Staph. aureus WT	+	+	-	-	+++
Staph. aureus 118	+	+	-	-	++
Staph. aureus A134	+	+	-	-	++
Staph. epidermidis 40	+	++	-	-	++
Staph. epidermidis RP62A	+	+	-	-	+++
Staph. lentus 84	-	-	-	-	+
E. coli DH5α	-	-	-	-	+++
Marine Vibrio (SB1)	-	+	-	-	-
Candida albicans	-	-	-	-	+++

(Inhibition zones: ≤10mm +, 11-15mm ++, >15mm +++).
SB (Sponge-associated bacteria); PB (Primmorph-associated bacteria).

Antibiotic resistance assay

The antibiotic resistance of the sponge isolates was tested against standard antibiotics. It was observed that the primmorphs associated bacterium PB2 was resistant to penicillin (150 μg ml^{-1}) and streptomycin (100 μg ml^{-1}).

Phylogenetic identification

Partial 16S rDNA sequences were obtained from PCR-products for phylogenetic identification (tab. 2). Although precise phylogenetic identification from partial sequences is limited, they provide sufficient information for the first assessment. The isolates SB1 and SB2 show species-level similarity (>98.0%) to the α-*Proteobacterium* MBIC3368.

Table 2 - Phylogenetic identification of sponge-associated and primmorph-associated bacteria.

Isolate	Method	Bases sequenced	Nearest phylogenetic neighbour	% similarity	Phylogenetic affiliation
SB1	PCR-product	465 bp	α-*Proteobacterium* MBIC 3368	98.9%	α-Proteobacteria
SB2	PCR-product	398 bp	α-*Proteobacterium* MBIC 3368	98.5%	α-Proteobacteria
SB6	PCR-product	379 bp	*Idiomarina ioihiensis*	98.9%	γ-Proteobacteria
PB1	cloned	1453 bp	Unidentified *Pseudomonas* sp.	94.5%	γ-Proteobacteria
PB2	cloned	1458 bp	Unidentified *Pseudomonas* sp.	94.4%	γ-Proteobacteria

SB (Sponge-associated bacteria); PB (Primmorph-associated bacteria).

Interestingly, this bacterial strain dominates the culturable microbial community of the Australian sponge *Rhopaloeides odorabile* (Webster & Hill, 2001). This bacterial strain has also been isolated from the Mediterranean sponge *Aplysina aerophoba*, which displayed antimicrobial activity against various gram-positive and gram-negative bacteria (Hentschel *et al.*, 2001). Denaturing Gradient Gel Electrophoresis (DGGE) of the microbial community of *A. cavernicola* following *in situ* transplantation reveals a transient DGGE band that shows highest sequence similarity to strain MBIC3368 (Thoms *et al.*, submitted).

Additionally, a Genbank entry reports on the isolation of the α-*Proteobacterium* MBIC3368 from an unidentified sponge (Genbank Accession Number AB012864). Apparently, the α-*Proteobacterium* MBIC3368 is frequently associated with diverse marine sponges irrespective of their taxonomic identity, geographic location or natural products profile. 16S rDNA sequence analysis of the isolate SB6 revealed species-level similarity to *Idiomarina ioihiensis* (Alteromonadaceae) (98.8%). Marine Alteromonads are well-known and abundant producers of antimicrobial compounds (Holmström & Kjelleberg, 1999; Nair & Simidu, 1987).

The 16S rDNA genes of both the primmorph isolates were cloned, sequenced nearly completely (>1350 bp) and deposited in Genbank (Preliminary accession numbers bankit 446266 (PB1) and bankit 454454 (PB2) (tab. 3). Considering that the genus *Pseudomonas* is phylogenetically well characterized, the sequence similarities to the nearest phylogenetic neighbour are remarkably low (94-95%). Interestingly, the construction of 16S rDNA libraries from aquarium maintained *S. domuncula* revealed only one sequence type which also belonged to the genus *Pseudomonas* (Böhm *et al.*, 2001). This study provides further evidence that *Pseudomonas* bacteria may be symbiotically associated with *S. domuncula* sponges. These isolates PB1 and PB2 represent possibly novel *Pseudomonas* species.

Conclusion

The current study shows that bacterial isolates from the sponge surface as well as from the primmorphs possess bioactive properties. Amongst all, the bacterium PB2 displayed potential antibacterial, antifungal, haemolytic and cytotoxic activities. It was also found to be resistant to the antibiotics and was identified as possibly a novel *Pseudomonas* sp. Thus, this investigation highlights the importance of sponge *S. domuncula* associated bacteria as a valuable resource for the discovery of novel bioactive molecules.

Acknowledgements

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Cytotoxic, cellular and antiviral activities of Pacific oyster, *Crassostrea gigas*, tissue extracts

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Shellfish farming is a very important economic activity all over the Atlantic coast, especially in Brittany and in Charente region with Marennes-Oléron area. But shellfish farming is also important along the Mediterranean coast. It involves fifty four thousand concessions, nineteen thousand hectares and thirty thousand workers. In that way, shellfish farming has an ecological impact as well as an activity of major economic interest for private and public authorities. That is why investigations are developed on the topic in fundamental and applied research. According to FAO data, aquatic production (including plants) has steadily increased since 1984. In 1996, total world production of finfish and shellfish from capture fisheries and aquaculture reached 120 million tons. The annual contribution of cultured species to total finfish and shellfish productions rose from 13% in 1990 to 22% in 1996. However, high density culture conditions induce a confinement of aquacultured population and an increase of density of those populations. In those conditions, we also observe more and more the presence of polluants and potential pathogens. A resettlement of animal between regions and between countries are also induced. Aquacultured animals under those stress conditions may generally present reduced immune defences and are more susceptible to infection. In these conditions, emergence and dissemination of diseases may be observed such as virus infection.

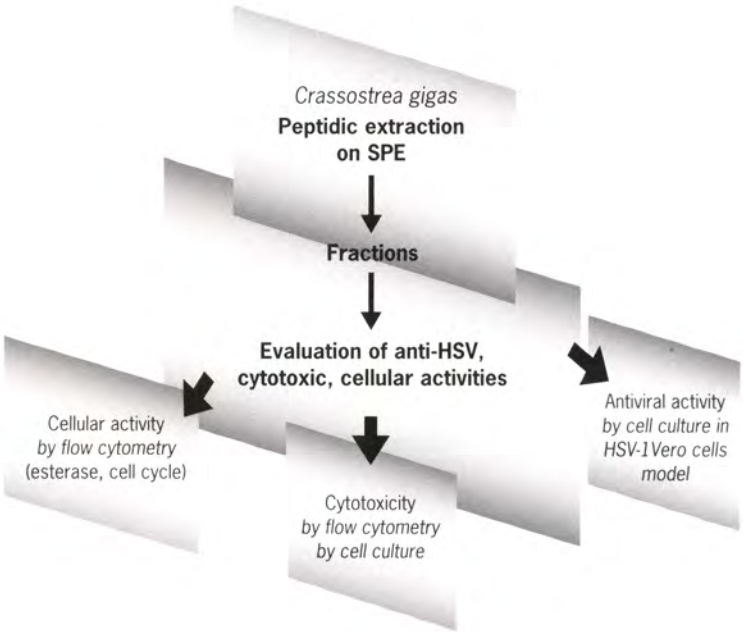
Little is known about the ability of invertebrates to control viral infections with antiviral substances (Pan *et al.*, 2000). Innate immunity has been investigated in molluscs. The immune system in marine bivalves is a non-adaptative and a non-specific system. Marine bivalves were found to develop a large variety of cellular defence mechanisms such as phagocytosis, aggregation, apoptosis or adhesion (Roch, 1999). It also involves soluble molecules like cytokins, hydrolytic enzymes or antimicrobial peptides. In comparison with vertebrates, invertebrates do not produce specific antibodies (Schapiro, 1975) and therefore, may rely on innate defence for host protection against virus (Schnapp *et al.*, 1996). At least some of innate immune pathways are well-conserved between invertebrates (such as drosophila) and mammals, suggesting that discoveries are applicable to a wide range of phyla.

Several viral diseases have seriously affected aquaculture industry. The discovery of viruses in marine bivalve is fairly recent event. Viruses morphologically similar to member of Herpesviridae have been identified in various marine bivalves species around the world. The first identification was reported in adult eastern oyster *Crassostrea virginica* and described as a member of the Herpesviridae (Farley *et al.*, 1972). Since, herpes virus have been detected in different oyster species such as Pacific oyster, *Crassostrea gigas* (Hine *et al.*, 1992; Nicolas *et al.*, 1992; Renault *et al.*, 1994), and European flat oyster, *Ostrea edulis* (Comps & Cochennec, 1993; Renault *et al.*, 2000). The virus infecting oyster has been named Oyster Herpes Virus type 1 (OsHV-1). Some work succeed on virus purification, characterisation and cloning of genetic material. OsHV-1 seems to be a novel member of Herpesviridae family. It is an enveloped virus with a linear double stranded DNA. Infections were associated with high seasonal levels mortality rates in larvae and juveniles in *Crassostrea gigas* and in *Ostrea edulis* species. However, non-adult Pacific oyster *Crassostrea gigas* mortality due to herpes virus has been reported in France. This may indicate that adult oyster could be more resistant to viral infections than a younger stage of development.

Data and literature about molecule with antiviral activity in *Crassostrea gigas* do not exist. But several studies have demonstrated proteins involvement in the non-specific host defence response in invertebrates and have been characterised. A number of them includes antimicrobial peptides (Gotz & Boman, 1985; Cociancich *et al.*, 1994; Destoumieux *et al.*, 1997; Bulet *et al.*, 1999; Mitta *et al.*, 1999; Relf *et al.*, 1999). Many reports have been published about *in vitro* antiviral effects of extracts, identified as peptides, from other aquatic species (Li *et al.*, 1965; Pani *et al.*, 1991; Aboudy *et al.*, 1994; Hamann *et al.*, 1996; Toreilles *et al.*, 1996; Yasin *et al.*, 2000). The main objective of this research programme is to provide knowledge of anti-herpes virus innate immunity in Pacific oyster, *Crassostrea gigas*, in order to develop new approchoaches for the control of viral infections.

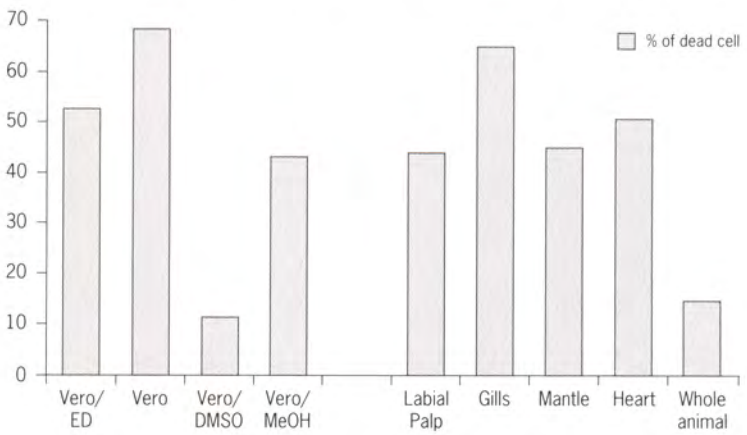
In order to isolate molecule with antiviral activities, we prepare different samples with different parameters such as seasonal variation. A winter set and a summer set have been prepared because of the oyster summer mortalities. Then we prepare a fed and non-fed set of animal to provide that our extract activity is not due to oyster diet like microalgae or bacteria. Different tissues have constituted different set in order to isolate molecules in a specific organ: heart, labial palp, mantle, gills, the whole animal and haemolymph. After a peptidic extraction on solid phase extraction (SPE) (fig. 1), of those different samples, fractions have been evaluated for their anti-herpetic activities by cell culture using a HSV-1 and Vero cell model, cytotoxic activities by flow cytometry and by cell culture and cellular activities by flow cytometry.

Figure 1
Research strategy.



The cellular activities (fig. 2) have been evaluated by flow cytometry in Vero cell at 4 and 72 hours. The result indicate that all cells treated with different extracts from labial palp, mantle, heart and the whole animal, show a lower rate of dead cell than the control, Vero cell only. Among all extract, the one from the whole animal shows the lowest rate.

Figure 2
Cell activity.



Cytotoxicities activities of our different extract have been evaluated by cytometry and by cell culture. By cell culture, those extracts seem not to be cytotoxic with a rate concentration in protein below 200 µg/ml. There are no seasonal and non-diet variation. By flow cytometry, we confirm those results. Furthermore, we suspect a cell stimulation for cell treated with extract from the whole animal, labial palp and gills. To evaluate antiviral activity, we do not have oyster cell culture and the infection by OsHV-1 on other cell-line had failed. Consequently, to test antiviral activities of our fraction we used the model of Vero cell infection by HSV-1. First, fractions have been evaluated on a multiplicity of infection (MOI) of 0.01. Tests have been observed at 24, 48 and 72 hours. But this has met with no success. We decide to work with a lower MOI of 0.001 with extract from the whole animal only. The result show that at 100 µg/ml of protein, there are no cytotoxic activity with 6% of cell destruction and 61% of cell protection against herpes virus. At 48 hours, we also suspect a cell stimulation from this extract on Vero cell. This observation confirms what we noticed by flow cytometry.
In order to identify molecules responsible of those effects the whole animal extract was applied to C18 HPLC. Different fractions have been collected and they have been evaluated for their antiviral activities by cell culture. At the MOI of 0.001, 4 purified fractions over 9 seem to have an antiviral activity.
The perspectives are to test by flow cytometry, purified fraction on Vero cell and haemocytes for their cytotoxicity and their cellular effect. After a characterisation at biochemical level of isolated molecule, we wonder to understand by molecular biology, how active molecules are synthetized in oyster organism. Anti viral activity research in haemolymph without treatment is going on.

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Chapter II

Aquaculture

Expression in *E. coli* and purification of the sea bass (*Dicentrarchus labrax*) interleukin-1β, a possible immuno-adjuvant in aquaculture

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Abstract

Interleukin-1β (IL-1β) is a pleiotropic cytokine that plays a pivotal role in regulating immune responses and shows a wide range of biological activities. Consistent with a central role in host defence, IL-1β has been postulated as an immuno-adjuvant. This molecule was extensively studied in mammals and in recent years many studies have confirmed the presence of functional IL-1β homologues in fish. Our group has cloned IL-1β from sea bass (*Dicentrarchus labrax*), the main Mediterranean aquacultured seawater fish species. The cDNA is 1292 bp and codes for a deduced peptide of 29.4 kDa with a pI of 5.1. As for trout and carp IL-1β precursor sequence, no candidate cut site for ICE enzyme was apparent in the alignments of sea bass IL-1β with other mammalian IL-1βs. Nevertheless, a possible mature peptide could start at Ala^{86}, giving a protein of 176 aa (with a MW of 19750.17), larger than that seen in mammals. By RT-PCR we have added to the nucleotide sequence coding for this polypeptide *Sal I* and *Sph I* restriction sites and the construct was cloned into a *Sal I*/*Sph I* cut pQE-30 expression vector. The plasmid was transformed in *E. coli* and the recombinant protein was partially purified with metal-affinity chromatography in non-denaturing conditions. The rIL-1β has been tested for the induction of phagocytosis to study its biological activity.

Introduction

Interleukin-1β (IL-1β) is a pleiotropic cytokine that plays a pivotal role in regulating immune responses and shows a wide range of biological activities centrally involved in the genesis and maintenance of inflammatory responses (Dinarello, 1997). This molecule was extensively studied in mammals where it is produced as a precursor molecule cleaved by the enzyme ICE, a caspase, to give the "mature" protein biologically active. Some of its fundamental roles include the activation of T and B-cells (Smith *et al.*, 1980), the activation of natural killer cells (Kullberg & Van der Meer, 1995) and the stimulation of macrophages.

Consistent with a central role in host defence, IL-1β has been postulated as an immuno-adjuvant.

In recent years, many studies have confirmed the presence of functional IL-1β homologues in fish (Clem *et al.*, 1985; Verburg van Kemenade *et al.*, 1995). The first non-mammalian IL-1β gene was cloned in rainbow trout (Zou *et al.*, 1999), followed by the cloning of IL-1β in plaice and in carp (Engelsma *et al.*, 2001). Recently, the availability of recombinant IL-1β molecules allowed the first studies of IL-1β "*in vitro*" biological activities (Hong *et al.*, 2001; Peddie *et al.*, 2001) and of their use as an "*in vivo*" immuno-adjuvant in fish vaccination (Yin & Kwang, 2000).

Our group has recently cloned IL-1β from sea bass (*Dicentrarchus labrax*), one of the main mediterranean aquacultured seawater fish species (Scapigliati *et al.*, 2001). The cDNA is 1292 bp and codes for a deduced peptide of 29.4 kDa with a pI of 5.1. At nucleotide level, sequence analysis showed highest similarity with rainbow trout (64.0%), carp (49.5%) and chicken (48.3%) interleukin-1β, whereas similarities of the deduced peptide sequences were 62.7% for trout, 46.0% for *Xenopus* and 45.5% for carp. As for trout (Zou *et al.*, 1999) and carp IL-1β precursor sequences, no candidate cut site for ICE enzyme was apparent in the alignments of sea bass IL-1β with other mammalian IL-1βs. Nevertheless, the alignments show that a possible mature peptide could start at Ala^{86}, giving a protein of 176 aa (with a predicted MW of 19750.17), larger than that seen in mammals. In this paper, we describe the production in *E. coli* of the recombinant protein, starting from Ala^{86}, its purification and the beginning of its bioactivity testing. Moreover, we are planning the successive use of the recombinant molecule as an immuno-adjuvant in sea bass vaccination experiments.

Material and methods

Sea bass IL-1β putative mature peptide cloning

Total RNA isolation was performed with Tripure™ Isolation Reagent (Roche) from approximately $5x10^7$ head kidney leucocyte cells previously stimulated with *E. coli* serotype 055:B5 lipopolysaccharide (LPS, Sigma), since the expression of IL-1β is known to be up-regulated by LPS, and purified over a discontinuous density Percoll gradient (Scapigliati *et al.*, 2001). By RT-PCR, using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, UK), we added to the cDNA encoding the putative mature IL-1β peptide *Sal I* and *Sph I* restriction sites using as forward primer: 5'-CAACTGCATGCGCGCAGTACAGCAAGCGAC and as reverse primer: 5'-GTGCAGGTCGACTTACTGTCCATTCAAAAGG. PCR amplifications were performed for 1 cycle of 5 min at 94°C, 35 cycles of 45 sec at 95°C, 45 sec at 58°C, 45 sec at 72°C, with a final extension step of 10 min at 72°C. All reaction mixtures were overlaid with mineral oil. PCR reactions were conducted using the MiniCycler™ model PTC-150-16 (MJ Research, USA) and amplified products

were visualised on a 1% agarose gel containing ethidium bromide (10 ng ml^{-1}), using 123 bp ladder (Sigma) as size marker. The PCR fragment obtained was purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK) and was directly sequenced on a ABI 377 automated sequencer (Applied Biosystems, UK). The construct was cloned into a *Sal I/Sph I* cut pQE-30 expression vector (Qiagen, West Sussex, UK), which adds a 6X His tag to the N-terminus of the protein, fundamental for the successive detection and purification of the expressed recombinant peptide. The pQE-30 plasmid was transformed into *E. coli* strain JM109 and the bacteria were grown in plates with Luria-Bertani (LB) and agarose medium containing 100 mg/ml ampicillin. Clones containing the insert were selected by PCR screening, plasmid DNA purified (Miniprep kit, Promega) and sequenced to confirm no alteration of the coding domain.

Time course recombinant sea bass IL-1β expression in *E. coli* and western blotting detection

A single colony harbouring the plasmid was cultured in 100 ml of LB medium containing ampicillin and grown, at 37°C and 250 g, until the OD_{600} was between 0.5-0.6 and, then, the expression of recombinant IL-1β was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 2 mM (Studier *et al.*, 1990). To perform a time course expression, 1 ml samples of the culture were taken at 1 hour intervals after induction from time 0 (no induction) to 6 hours. The cells were harvested by centrifugation for 1 min at 15000 g and stored at -20°C after discarding the supernatants. Successively, the samples were dissolved in 50 ml of loading gel buffer containing sodium dodecyl sulphate and β-mercaptoethanol, boiled for 5 min, and then 10 ml loaded on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli, 1970), with High-Range Rainbow Molecular Weight Markers (myosin 220 kDa, phosphorylase b 97 kDa, albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 20 kDa, lysozyme 14 kDa) (Amersham Pharmacia Biotech, UK) as molecular weight markers.

The expressed protein was detected by western blotting with an antibody against the RGS-4X His epitope using the QIA*express* Anti-His HRP Conjugate Kit (Qiagen, West Sussex, UK). After the electrophoresis, proteins were blotted on a nitrocellulose membrane (Schleicher and Schuell BA85, Germany) and the immunodetection was performed with the anti RGS-4X His antibody (diluted 1/1500) conjugated with horseradish peroxide. To visualize the detected protein a chromogenic method with 4-chloro-1-naphthol was used.

Sea bass recombinant IL-1β (rIL-1β) purification

For sea bass recombinant IL-1β purification, a single colony harbouring the plasmid was cultured in 100 ml of LB medium containing ampicillin and grown overnight at 37°C and 250 g. Successively, 20 ml of this culture was placed in 500 ml of fresh LB medium containing ampicillin for 4 hours and the expression of recombinant IL-1β was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 2 mM (Studier *et al.*, 1990). The *E. coli* cells were harvested after 1 hour of induction by centrifugation at 4°C and 800 g, resuspended in 6 ml of lysis buffer (50 mM Tris-Cl, pH 8, 500 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40) (Hong *et al.*, 2001) and sonicated on ice to collect the recombinant protein.

The protein was purified with metal-affinity chromatography on columns with nickel-nitrilotriacetic acid (Ni-NTA) matrices (Qiagen) specific for biomolecules which have been tagged with histidine residues. The cell lysate was applied to a column equilibrated with the lysis buffer and, after elution of the proteins lacking the His tag with 20 mM imidazole, the recombinant protein was detached from the resin using 50 mM imidazole. The purified protein has been checked on 12% SDS-PAGE and the concentration determined by the Bradford method (Bradford, 1976).

Bioactivity testing

The biological activity of the rIL-1β was tested on head kidney leucocytes cells purified using a discontinuous density Percoll gradient as described by Scapigliati *et al.* (2001). For the phagocytosis assay the cells were adjusted to 16×10^6 viable leucocytes per millilitre of L-15 (Gibco) medium. Culture slides (Becton Dickinson), divided in 8 different chamber polystyrene vessels containing 250 ml of the cellular suspension (about 4×10^6 cells) each, were incubated with 0, 1, 10, 30 and 100 ng/ml of rIL-1β for 4 hour at 18°C. Each treatment was applied in triplicate. Then, the slides have been washed with Hanks Balanced Salt Solution (HBSS, Gibco) to remove the non-adherent cells and flooded with 250 ml of a denatured yeast suspension (*Saccharomyces cerevisae*, 4.8×10^5 cells per millilitre of L-15 medium) made fluorescent using fluorescein-5-isothiocyanate (FITC, Molecular Probes). Phagocytosis were allowed to occur for 1 hour at 18°C in a humidity chamber before excess yeast was washed off with HBSS and the slides air dried. Following methanol fixation, the preparations were stained sequentially with May-Grunwald (BDH) and Giemsa (BDH) stains. Slides were viewed under oil immersion at x 100 on a microscope. Approximately 100 cells were counted in random fields of view, recording the number of phagocytosed yeast cells and the number of yeast particles phagocytosed per cell (Crampe *et al.*, 1997). Data were expressed as mean ± S.E. percentage phagocytosis and phagocytic index.

Contamination testing

To ensure that the rIL-1β used was inducing the effects evidenced during the study, the endotoxin (LPS) levels in the samples were determined with a *Limulus* amebocyte lysate kit (Bio Whittaker).

Results

Production and purification of rIL-1β in *E. coli*

The putative mature sea bass IL-1β molecule was expressed in *E. coli* cells after cloning the cDNA into the *Sal I/Sph I* restriction sites of pQE-30 expression vector (Qiagen, West Sussex, UK), this plasmid adds a 6X His tag at the N-terminus of the protein fundamental for the successive detection and purification of the recombinant peptide. Time course expression from 0 (no induction with IPTG) to 6 hours reveals that after 1 hour the recombinant protein is already expressed at the maximum (data not shown). Figure 1 shows, on the right side, the 12% SDS-PAGE analysis of the whole cell lysate before and after induction with IPTG (1 and 2 hour induction) and, on the left side, the corresponding immuno-blotting using the antibody against the RGS-4X His epitope to detect the expressed recombinant protein. The level of expression is the same in the two lanes where the rIL-1β is evidenced.

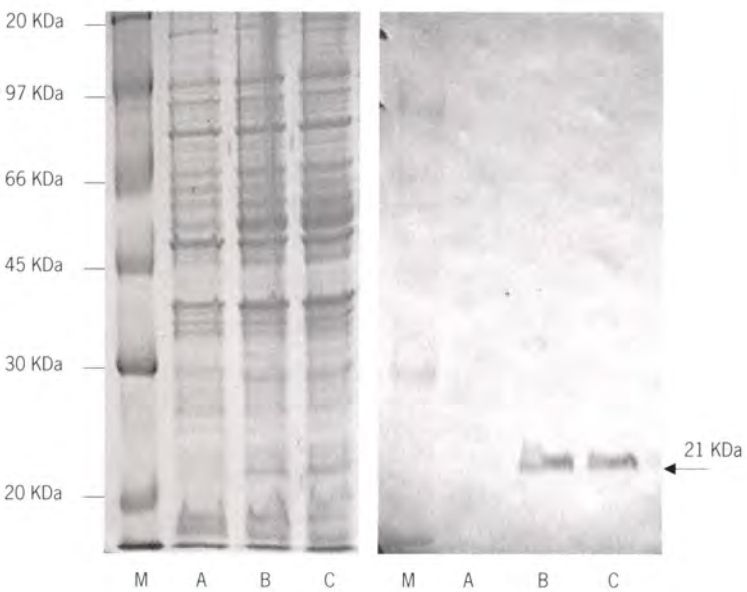

Figure 1
On the left side of the figure a 12% SDS-PAGE loaded with the molecular weight markers (lane M), the total lysate cells before induction with IPTG (lane A), the total lysate after 1 and 2 hour of induction with IPTG (lane B and C, respectively) is shown.
On the right side of the figure the immuno-blotting of the electrophoresis gel with the samples loaded in the same order as before is shown, the expressed recombinant protein has been evidenced with a specific antibody against the RGS-4X His epitope.

Figure 2 shows the SDS-PAGE analysis of the whole cell lysate and flow through after absorption to an Ni-NTA affinity column, as eluted with 20 and 50 mM imidazole. The final eluted fraction exhibited two bands, the lower one corresponds to a MW of approximately 21 kDa,

which is in close agreement with the MW of 21.3 kDa predicted from the amino acid sequence inferred from the cDNA sequence. This band is recognised in immunoblotting by the antibody against the RGS-4X His epitope (data not shown). The yield of the recombinant protein, as determined by Bradford method, was about 200 mg of purified protein from 500 ml of culture broth.

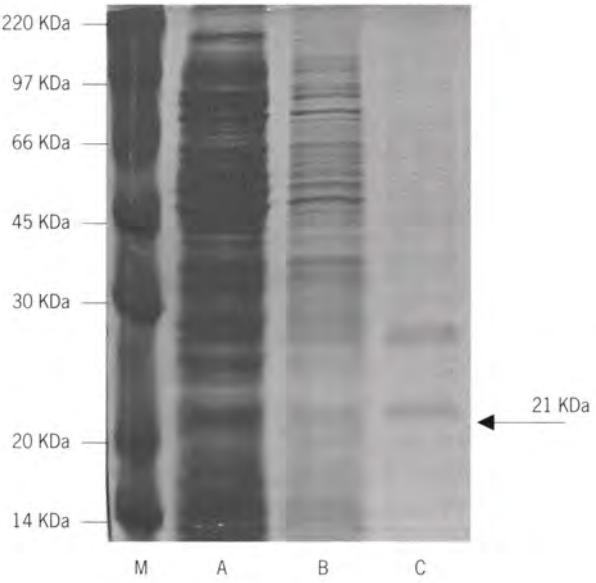

Figure 2
Partial purification of sea bass rIL-1β. 12% SDS-PAGE loaded with the molecular weight markers (lane M), the whole cell lysate (lane A) and flow through after absorption to an Ni-NTA affinity column as eluted with 20 (lane B) and 50 mM (lane C) imidazole.

Bioactivity of rIL-1β

The partially purified protein has been tested for its effects on cell function. During sea bass head kidney leucocytes phagocytosis assay, a clear stimulation was detected in the samples treated with the rIL-1β, whether expressing the data as percentage phagocytosis or as phagocytic index (fig. 3).

Macrophages were stimulated without (control) rIL-1β as a negative control, with the buffer used during the purification of the recombinant protein (an additional control) and with 1,10, 30 and 100 ng/ml of the peptide. Values of the percentage phagocytosis from cells treated with 1 ng/ml rIL-1β showed little variation in comparison with the controls. On the contrary, a clear stimulation was observed in samples treated with 10 and 30 ng/ml rIL-1β, whether the treatment with 100 ng/ml rIL-1β shows a down-regulation of the effect. Values of the phagocytic index from cells treated with 1 ng/ml rIL-1β showed little variation in comparison with the two controls. A clear stimulation, as before, was observed in samples treated with 10 and 30 ng/ml rIL-1β, whether the treatment with 100 ng/ml rIL-1β shows a down-regulation of the effect.

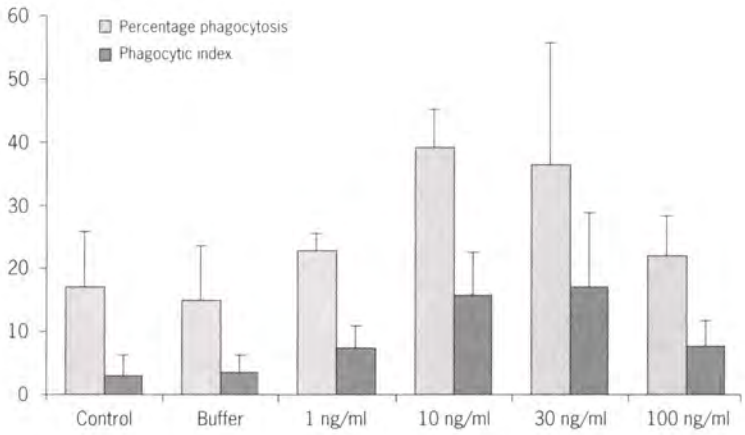

Figure 3
Effects of stimulation with varying concentrations of sea bass rIL-1β (1, 10, 30, 100 ng/ml) on percentage phagocytosis and phagocytic index on sea bass head kidney leucocytes. Macrophages were stimulated without (control) rIL-1β as a negative control and with the elution buffer used in the protein purification as an additional control. Data are presented as mean ± S.E. of triplicate samples, with each experiment performed at least twice.

Contamination test

According to the *Limulus* amebocyte lysate assay, the LPS content in the rIL-1β preparation was less than 20 ng (62.5 EU)/mg of protein. Thus, at the highest concentration of the recombinant protein used in these studies, the LPS concentration did not exceed 2 ng/ml.

Discussion

In the presented study, the predicted mature sea bass IL-1β has been expressed in *E. coli* and, after purification, its bioactivity has been studied "*in vitro*" using head kidney leucocytes. Both the percentage phagocytosis and phagocytic index showed significant dose-dependent increases. Moreover, the 100 ng/ml addition of rIL-1β seems to cause a down-regulation effect of the stimulation, probably as the IL-1β quantity is too high for the cells. The bacterial endotoxin contamination of rIL-1β preparation was low, tipically 0.2-2 ng/ml, and this is less than the amount considered to be the minimum able to induce IL-1β expression in trout head kidney leucocytes (Zou *et al.*, 2000).

In conclusion, we have started to study the biological activity of sea bass rIL-1β and the obtained data confirmed that this molecule is likely to be a proinflammatory mediator in fish and to have an impact on leucocytes responses. Further researches are in progress to complete the study of rIL-1β biological activity with other methods and, moreover, to verify the possible use of the protein as an immuno-adjuvant in aquaculture. Most aquaculture vaccine formulations contain adjuvants that augment the antigen-specific immune response in order to maximise protection against diseases and some of these adjuvants are recombinant proteins or peptides. Recombinant mammalian cytokines polypeptides, like IL-1β, have been proved to exhibit similar biological activities to those of native IL-1β (De Chiara *et al.*, 1986) and were subjected to vaccine tests (Bomford, 1998).

In fish, in contrast to the side-effects (pyrogenic effects) observed in mammals, the use of rIL-1β should not cause physiological problems, as it was shown in carp (Yin & Kwang, 2000), and, therefore, the potential application of this molecule as an immuno-adjuvant in vaccination is even more achievable.

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Identification of genes expressed in the liver of the Atlantic salmon (*Salmo salar*)

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Abstract

An investigation of the genes expressed in the liver of adult Atlantic salmon was undertaken using the expressed sequence tag (EST) strategy. A representative cDNA library was constructed and assessed for quality, and then screened with a total cDNA probe to identify cDNA clones encoding both abundant and rare mRNA transcripts. 733 ESTs, representing the 5'-termini of 170 abundant and 563 rare mRNA encoding cDNA clones respectively, were determined. Subsequent cluster and bioinformatic analyses of these ESTs resulted in the identification of 93 genes, of which only 7 had previously been reported in Atlantic salmon. Classification of these genes showed that 33 (36%) encoded proteins associated with primary liver functions, i.e. transport, acute phase response and blood clotting. Finally, comparative analysis revealed that 12 of the 16 genes shown to encode abundant mRNA transcripts in salmon liver have homologues that have also been shown to be highly expressed in mammalian liver systems.

Introduction

The liver is an important organ playing a vital role in carbohydrate, lipid, steroid, amino acid and prostaglandin metabolism, in detoxification and in serum-protein and biliary acid production. In mammals, the unique liver cell types are the hepatocytes which compose approximately 76% of liver cells, and the biliary and sinusoidal cells (Blouin *et al.*, 1977). The hepatocyte performs most of the known liver functions including the production of plasma proteins (i.e. transport, blood clotting and acute response proteins), detoxification and ATP production (Feldmann, 1994). In mammals, the liver is a model tissue for gene expression studies with gene regulation being primarily exerted at the transcriptional level (Derman *et al.*, 1981).

By comparison, less is known of liver gene expression in fish. Also, with respect to the anadromous Atlantic salmon, the liver is associated with the parr-smolt transformation as evidenced from studies showing hepatic ultrastructure change (Robertson & Bradley, 1991), increase hepatic enzyme activity (Blake *et al.*, 1984), and alterations in fatty acid content (Sheridan, 1989). Analysis of liver gene transcription associ-

ated with smoltification is limited to individual genes (Hardiman *et al.*, 1994) reflecting the current paucity of identified salmon genes. This study reports an EST-based identification of genes expressed in the liver of adult Atlantic salmon. After construction and quality assessment of a liver cDNA library, 960 selected cDNA clones, composed of 192 abundant and 768 rare mRNA encoding cDNA clones, were used for 5'-terminus EST determination. Gene identification was based on homology searches of the NCBI nr protein database. Finally, the identified salmon genes were classified according to cellular role and compared to similar data from mammalian liver systems.

Material and methods

cDNA library construction and quality assessment

Equivalent amounts of liver tissue was dissected from one male and one female, approximately 3 kg, adult Atlantic salmon (MOWI strain) obtained from an Irish fish farm. The method details for the construction and quality assessment of the liver cDNA library have previously been described (Davey *et al.*, 2001).

cDNA clone identification, EST determination and bioinformatic analysis

Abundant and rare mRNA encoding clones were identified by screening aliquots of the cDNA library with a liver total cDNA probe (Martin *et al.*, 2002). Hybridisation-positive and -negative plaques, representing abundant and rare mRNA encoding cDNA clones respectively, were catalogued and stored separately. EST sequencing was performed on 960 selected salmon liver cDNA clones (192 abundant and 768 rare mRNA encoding) targeting the 5'-termini of the respective cDNA fragments. Vector sequences were removed and all ESTs > 270 bp in length were aligned using the Clustal X program (Thompson *et al.*, 1997). The longest EST from each cluster was used to screen the NCBI nr protein database (Benson *et al.*, 1998) using the Blast X program located on the NCBI Blast homepage (*http://www.ncbi.nim.nih.gov/BLAST/*). Each unique EST has been deposited in the GenBank dbEST under accession numbers BI468016-BI468193, BI544051-BI544053 and BI544216-BI544217. Full method details for the EST determination and bioinformatic analysis have previously been described (Martin *et al.*, 2002).

Results and discussion

EST selection and Atlantic salmon gene identification

Based on the selection criteria, 170 abundant and 563 rare mRNA class ESTs were determined. The subsequent cluster analysis revealed that the 733 liver ESTs were composed of 246 unique ESTs, derived from 128 EST clusters and 118 singletons, and 68 and 178 of which represented

abundant and rare mRNA transcripts respectively. Due to the relatively low number of fish gene sequences in the international databases, a protein-based homology strategy was used to screen for homologous genes. The criterion for salmon gene identification was > 50% identity over a contiguous series of > 50 amino acids. The results showed that 117 (48%) of the Atlantic salmon ESTs found matches of sufficient homology. After accounting for redundancy whereby different ESTs corresponded to different regions of the same mRNA transcript, the 117 ESTs identified 93 different salmon genes. The 93 identified genes, composed of 23 abundant and 70 rare liver mRNA encoding genes, included 7 genes previously described in Atlantic salmon and 25 in salmonids. A list of these genes, the respective ESTs and their database accession numbers, and the best match and homology values are presented in Martin *et al.* (2002). In contrast, the 129 (52%) unidentified liver ESTs were composed of 104 ESTs that did not reach the gene identification criterion, 7 ESTs which did but to as yet unidentified mouse and human genes, and 18 ESTs which showed no database match.

Classification and comparative analysis of identified Atlantic salmon genes

The 93 identified salmon genes were classified according to cellular role by comparison with the more comprehensive mammalian liver gene expression data available (Kawamoto *et al.*, 1996). Primary liver functions accounted for 33 (36%) of the 93 identified salmon genes and included genes encoding plasma proteins, protease inhibitors, coagulation factors, complements, lipoproteins, detoxificants and enzymes involved in glycolysis and gluconeogenesis (tab.). A further 45 (48%) salmon genes encoded proteins involved in more general cell functions (e.g. ribosomal proteins, cell communication, mitochondrion-encoded, metabolism and housekeeping), while 15 (16%) genes could not be classified.

Identified Atlantic salmon genes associated with primary liver functions.

Liver function	Gene
Plasma proteins	β-globin, haptoglobin*, hemopexin, penraxin, sero-transferrin*, serum albumin*
Protease inhibitors	α1-microglobulin/inter-α-trypsin inhibitor*, antithrombin, heparin, inter-α-trypsin inhibitor, serine proteinase inhibitor
Coagulation factors	angiopoietin, β-fibrinogen*, γ-fibrinogen*, β-fibrin, prothrombin*, thrombin β-chain
Complements	chemotaxin, complements C3-1*, C3-3*, C4B*, C8β, C9, B/C2-B, Bf-1 and Bf-2, Orla C3-1 and C4
Lipoproteins	apolipoproteins A-I* and C-II*, lipoprotein lipase
Detoxificants	cytochrome P450
Glycolysis and gluconeogenesis	triosephosphate isomerase

* Denotes genes shown to highly expressed in salmon and mammalian liver tissues.

Sixteen of the 33 genes associated with primary liver functions encoded abundant mRNA transcripts in salmon liver. Comparison with mammalian liver gene expression data revealed that 12 of these salmon genes have homologues that also showed strong mRNA expression profiles in mouse and human liver (tab.). This similarity suggests that many components of the primary liver functions have been conserved since the teleost fish-tetrapod divergence 400-450 million years ago. It also indicates that the range of salmon liver ESTs identified in this study should be useful to monitor many facets of liver gene expression under different physiological conditions, e.g. smoltification, development, pathogen infection.

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Effect of various sources of carotenoids on survival and growth of goldfish (*Carassius auratus*) larvae and juveniles

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Abstract

Two separate trials were performed to evaluate the effects of adding various dietary sources of carotenoids on survival and growth of goldfish (*C. auratus*) larvae and juveniles. In the first trial, 2000 larvae (initial body weight: 3,4±0,7 mg) were randomly distributed in a closed water recirculated system and fed four microparticulate diets (MP). Diets were formulated to contain a constant level of pigments (45 mg kg^{-1}) from different sources of carotenoids and larvae were subjected to the following dietary regimes from first feeding to day 28: (1) an MP containing algae *Chlorella vulgaris* biomass (diet CV), (2) an MP containing cyanobacteria *Spirulina platensis* biomass (diet SP), (3) an MP containing synthetic pigment astaxanthin (diet AS) and (4) a control diet without added pigments (diet C). All treatments were tested in duplicate as well as a fasted control group. In the second trial, a fifth diet, containing *Haematococcus pluvialis* biomass (diet HM), was tes .ed. Diets were fed to homogenous groups of 45 juvenile *C. auratus* (1.7±0.1g), in triplicate. Water temperature was maintained at 25±1ºC during the 12 weeks long trial. High survival rates were recorded in both studies with the exception of the unfed control group of larvae for which total mortality was recorded at day 16. Furthermore, there were no significant differences among treatments, for either survival or growth. Our results demonstrate that survival and growth of goldfish larvae and juveniles were not affected by the inclusion of carotenoids (45 mg kg^{-1}) in the diet.

Introduction

Many compounds, such as betaine, inosine 5-monophosphate and amino acids have been shown to stimulate feeding in fish (Metailler *et al.*, 1983; Mearns, 1986; Knutsen, 1992; Kolkovski *et al.*, 1997) and are natural constituents of phytoplankton. Carotenoids, lipid soluble pigments, may be carried through the food chain from primary producers, namely microalgae, because animals cannot synthesize them *de novo* (Goodwin, 1984). A positive metabolic role of carotenoids in the nutrition of larval fish and for the survival rates of young fry was discussed (Shahidi *et al.*, 1998).

Numerous reports show that carotenogenic microalgae appear as suitable sources of carotenoids in fish feeds (Sommer *et al.*, 1992; Choubert & Heinrich, 1993; Gouveia *et al.*, 1998). The aim of this work was to determine the effect of supplementation of diets with carotenoids (natural and synthetic) on growth and survival rates of ornamental goldfish (*Carassius auratus*).

Material and methods

Fish, diets, microalgae, facilities and feeding protocol

In the first trial, larvae of goldfish (red Oranda variety) were obtained from broodstock in our laboratory by forced spawning induced following intraperitoneal injections of a crude extract of carp hypophysis (Rema, 2000). Larvae (mean initial length: 6.4±0.8 mm) were randomly distributed in 10 plastic tanks (200 larvae T^{-1}) in a closed water recirculated system (Charlon & Bergot, 1984). Four microparticulate diets (MP) were formulated to contain a constant level of pigments (45 mg kg^{-1}). Larvae were subjected to one of the following dietary regimes from first feeding (day 2 post-hatch) to day 28: (1) MP with algae *Chlorella vulgaris* (diet CV), (2) MP with *Spirulina platensis* (diet SP), (3) MP with synthetic pigment astaxanthin (diet AS) and (4) the microparticulate diet alone (diet C). The proximate composition of diets is presented in table 1. The MP control (diet C), based in yeast and fish protein hydrolysate, was routinely used in previous studies with ornamental cyprinids larvae in our experimental fish farm. An automatic feed dispenser (Charlon & Bergot, 1986) was used to feed the experimental diets with a particles size between 100 to 200 mm during the 1st week, between 200 to 400 mm during the 2nd week and between 400 to 630 mm during the 3rd and 4th weeks. All treatments were carried out in duplicate as well as a fasted control group. Photoperiod was set on a 12:12 h light/dark cycle throughout the study. The trial lasted 4 weeks and water temperature was kept at 25±1°C with a flow rate of 0.4 l $min.^{-1}$ $tank^{-1}$ during the 1st week and of 0.8 l $min.^{-1}$ from the 2nd week onwards. At days 7th, 14th, 21st and 28th samples of 10 larvae were taken from each tank, anaesthetized and the total length and weight recorded. At the end of the trial the larvae from each tank were fasted for one day, counted and total biomass was weighed.

The second trial was performed with juvenile goldfish (1.5 month old) raised in the UTAD experimental fish farm. Triplicate homogenous groups of 45 fish (initial mean body weight: 1.7±0,1g) were randomly distributed in 15 rectangular plastic tanks with a flow rate of 0.81min^{-1}. Water temperature was maintained at 25±1°C and artificial photoperiod (12L: 12D) was used during the 12 weeks trial. Five isonitrogenous diets were formulated to contain a constant level of pigments (45 mg kg^{-1}): biomass from algae *Chlorella vulgaris* (diet CV), *Spirulina platensis* (diet SP), *Haematococcus pluvialis* (diet HP), synthetic pigment

astaxanthin (diet AS) and a control diet without pigments (diet C). The microalgal biomass and synthetic pigments were finely ground (<800 mm) and mixed with a basal diet of commercial trout feed (Protein: 52% DM; lipid: 17% DM) and pelleted in a California Pellet Miller (CPM). Fish were fed by hand twice a day to apparent satiety, and daily feed intake was recorded. The microalgae *C. vulgaris*, *H. pluvialis* and cyanobacteria *S. maxima* were cultivated in raceways ponds, polyethylene bags and alveolar photobioreactors, respectively, using appropriate mediums (Gouveia *et al.*, 1996).

Table 1 - Composition of the experimental diets (dry weight).

Ingredients	Diet (g/kg dry weight)			
	C	AS	SP	CV
Microparticulate diet[1]	1000	999.5	992.5	988.8
Synthetic astaxanthin[2]	0	0.5	0	0
Spirulina platensis[3]	0	0	7.5	0
Chlorella vulgaris[4]	0	0	0	11.2
Proximate analysis*				
Dry matter (%)	91.6	91.4	91.8	92.0
Crude protein (%DM)	52.1	52.2	52.0	51.8
Lipid (%DM)	7.6	7.7	7.5	7.4
Energy (kJ g^{-1} DM)	19.6	19.3	19.2	19.0
Ash (%DM)	15.7	15.9	15.9	16.0

1. Microparticulate diet for cyprinid larvae (based on yeast and fish protein hydrolysate).
2. Pigments: 8 % DM; 3. Pigments: 0.6% DM; Protein: 25% DM; 4. Pigments: 0,4% DM; Protein: 65% DM.
*Values are means of 2 determinations.

Analytical methods

Chemical composition analysis of the diets was made using the following procedures: dry matter, by drying at 10 °C for 24 h; ash, by combustion at 550°C for 12 h; crude protein (micro-Kjeldahl; N x 6.25), fat by dichloromethane extraction (Soxhlet) and gross energy, in an adiabatic bomb calorimeter (IKA).

Statistical analysis

Data are presented as means ± standard deviation. Data from each trial was analysed independently. Data on survival and weight of larvae were transformed (angular and logarithmic, respectively). To test differences between dietary treatments, data were subjected to one-way analysis of variance and when appropriate, means were compared with the Newman-Keuls multiple range test. Statistical significance was tested at a 0.05 probability level. All statistical tests were performed using the Statgraphics (7.0) statistical package.

Results

At the end of the larvae trial, high survival rates were observed in all treatments (tab. 2) with the exception of the unfed control group with a total mortality recorded at day 16th. There were no significant differences among treatments ($P > 0.05$) in respect of total length and total weight of larvae, although a slight increase in both parameters was noticeable for treatment C.

Table 2 - Mean values of survival, total length and weight of larvae at the end oh the trial (day 28)*.

Parameter	C	AS	SP	CV
Survival (%)	98.9±0.1[a]	97.5±0.1[a]	98.6±0.1[a]	97.2±0.2[a]
Mean initial weight (mg)	3.4±0.7	3.4±0.7	3.4±0.7	3.4±0.7
D_{07}	17.0±4.6	17.6±4.6	18.3±5.1	17.6±5.2
D_{14}	47.7±11.5	39.4±5.7	39.3±7.2	41.5±9.4
D_{21}	126.9±30.2	99.5±30.8	109.5±32.1	113.5±34.1
D_{28}	245.1±22.0[a]	223.0±4.7[a]	221.2±6.2[a]	227.3±10.0[a]
Mean initial length (mm)	6.4±0.8	6.4±0.8	6.4±0.8	6.4±0.8
Mean final length (mm)	22.6±1.2[a]	20.6±0.7[a]	21.8±0.3[a]	22.1±0.3[a]

* Mean ± standard error (n = 20). In the same line, values with different superscript letters are significantly different ($P < 0.05$).

Table 3 reports the growth performance and diet utilization by juvenile goldfish. At the end of the trial high survival rates were observed in all treatments ($P > 0.05$). There were no significant differences among treatments as regards mean final weight and weight gain. The values for specific growth and feed conversion rates were homogeneous, and no significant differences were observed between treatments.

Table 3 - Growth performance and diet utilization by juvenile goldfish*.

Parameter	C	SP	HP	CV	AS
Mean initial weight (g)	1.6±0.1	1.7±0.1	1.7±0.1	1.7±0.1	1.7±0.1
Mean final weight (g)	4.3±0.03	4.4±0.03	4.4±0.1	4.5±0.1	4.3±0.1
Weight gain (%)[1]	173.6±10.3	161.3±7.3	159.4±10.9	167.4±11.6	152.9±5.9
Specific growth rate (%)[2]	1.2±0.04	1.1±0.03	1.1±0.05	1.2±0.05	1.1±0.03
Feed conversion ratio[3]	2.1±0.04	2.1±0.04	2.1±0.06	2.0±0.1	2.1±0.1
Feed efficiency (%)[4]	48.3±0.01	48.6±0.01	47.4±0.01	50.1±0.02	47.2±0.01
Mortality (%)	2.2	-	-	2.2	-

* Mean ± standard error.
1. Weight gain (%): (final body weight - initial body weight)/initial body weight) 100.
2. Specific growth rate (%): (Ln final weight - Ln initial weight) 100/time (days).
3. Feed conversion ratio: (feed intake DM/weight gain).
4. Feed efficiency (%): (1/feed conversion ratio).

Discussion

Astaxanthin and canthaxanthin are widely used as dietary supplements in diets for salmonids as a method for inducing the typical pink colour of their flesh (Torrissen, 1985; Choubert & Storebakken, 1989; Skrede *et al.*, 1990). Besides the effectiveness of carotenoids in pigmentation of fish skin a positive effect in the nutrition of larval fish and survival rates of young fry was discussed (Shahidi *et al.*, 1998). The addition of algae to the rearing tanks of marine fish larvae (green water culture) has been shown to enhance growth and survival as well as the quality of the fry (Reitain *et al.*, 1997; Lazo *et al.*, 2000). One of the beneficial effects attributed to adding algae is an increase in ingestion rates of food by marine fish larvae (Naas *et al.*, 1992). The main objective of our study was to evaluate the effects on growth and survival of adding carotenoids in the diet and we used the same concentration values (45 mg kg^{-1} diet) referred by others authors in pigmentation studies.

The results obtained with goldfish larvae are in accordance with previous trials at UTAD experimental fish farm (Rema, 2000) and the larvae grew within the range normally found in the literature data for this species (245 mg; 28 days). The high nutritional value of the microparticulate diet C for rearing this particular goldfish larvae variety seems to be one of the major observations in this study.

Despite the long duration of the second trial, the results evidenced not sound effect of dietary carotenoids upon the growth, or the survival, of juvenile goldfish. In fact, the fish grew within the range normally found in the literature data and neither growth nor feed efficiency was significantly affected by inclusion of carotenoids in dietary treatments. No mortality was associated to the experimental treatments and it is worth mentioning that the rearing conditions, namely water parameters and UV-sterilization, seem to be very effective in the rearing of the fish. The inclusion of 45 mg carotenoids in the diet, besides this effectiveness on skin pigmentation (visual observation), was not sufficient to induce any differences in growth and survival.

Our results suggest that the algal supplement to the basal diet apparently produces no significant alterations in growth and survival rates, independently of the alga species, in both trials.

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Chapter III

Biopolymers

Fucose-based polysaccharides of marine origin: research tools with therapeutic potential

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Introduction

Fucans are polymers of fucose; usually but not exclusively α-L-fucopyranose. The first fucans to be described were extracted from brown algae, and termed fucoidins (later fucoidans). In the literature both names, fucan and fucoidan, have been used to refer to fucose polymers of whatever origin; but in this article the term fucan will refer to any fucose polymer (in line with IUPAC recommendations) with the name fucoidan reserved for the complex, fucose-rich polysaccharides of brown algae.

The effects of fucoidan on mammalian systems have been recognised for many years; for example, their anticoagulant and antithrombotic activity, capacity for immunomodulation, use as selectin ligands and macrophage scavenger receptor ligands, properties as modulators of fertilisation in mammals, and effects on cell proliferation have all been documented in the literature for some time.

Structural studies, however, have been much more limited. The complexity and heterogeneity of fucoidans have hindered analytical and spectroscopic studies. Percival & McDowell (1967) came to the conclusion in 1967 that fucoidans consisted of a 2-linked polyfucose backbone with side-chains and sulphate substitution at the remaining 3- and 4-positions. Patankar *et al.*, (1993) had more sophisticated techniques available, and concluded that the fucose backbone was in fact 1-3 linked, with 2- and 4-linked sulphate and fucose branches. Classic techniques such as methylation analysis are difficult to apply to sulphated fucans of any type; it is not easy to distinguish between positions of sulphation and positions of glycosidic linkage by this method.

The fucans of marine invertebrates

Mourão and co-workers have isolated and characterised several fucans from echinoderms; one from the body wall of sea cucumber (Ribeiro *et al.*, 1994) and several others from sea urchin egg-jelly (Mulloy *et al.*, 1994; Alves *et al.*, 1997,1998; Vilela-Silva *et al.*, 1999, 2002; Pereira *et al.*, 1999). Analytical results (such as sulphate content and methylation analysis) were similar to those for the plant-derived fucoidans, but NMR spectroscopy revealed spectra of a simplicity which clearly indicated a

regular repeating structure with little or no heterogeneity (Mulloy *et al.*, 1994). It became clear that the egg-jelly of each species of sea urchin has its own specific sulphated fucan, in which the repeating pattern of up to four monosaccharide units in size was defined solely by the pattern of sulphation (fig. 1). The linkage position varies from species to species but is (in all species so far studied) constant within each species; all the compounds so far studied have been unbranched.

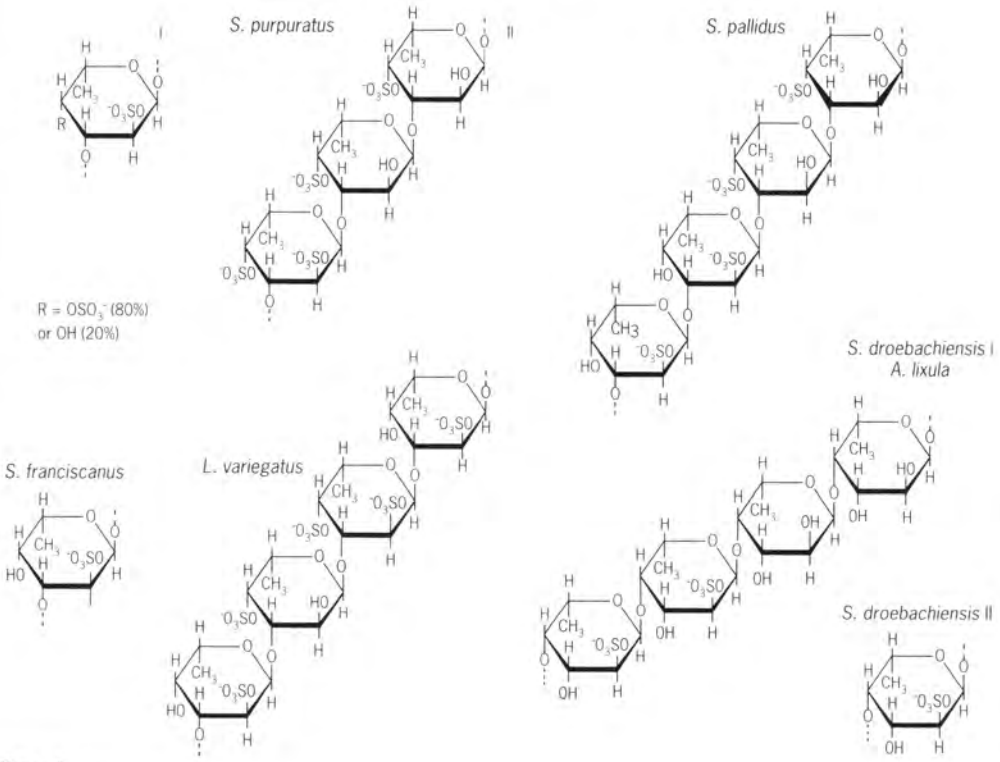

Figure 1
Structures of regular repeating fucans from the egg-jelly of several sea urchin species. The fucans from *Strongylocentrotus purpuratus*, *S. pallidus*, *S. franciscanus* and *Lytechinus variegatus* are all 3-linked α-L-fucans and can be distinguished from each other only by sulphation pattern. The fucans of *Arbacia lixula* and *Strongylocentrotus droebachiensis* are 4-linked α-L-fucans. Two species, *S. purpuratus* and *S. droebachiensis*, produce two different fucans, though individual females produce only one of the two possible fucan types.

The physiological significance of this structural variation lies in the mechanism by which sea urchins ensure species specificity in fertilization. Sea urchin spermatozoa must undergo a morphological change known as the acrosome reaction in order to fertilise the egg successfully, and this reaction is stimulated by the egg-jelly fucan of their own species only. This phenomenon demonstrates a high degree of specificity modulated entirely by the sulphation pattern of a linear polysaccharide.

The lack of heterogeneity of these fucans is in sharp contrast to the algal fucoidans. An apparent example of heterogeneity was seen in the fucan from the egg-jelly of *S. purpuratus*; but this was found to be a mixture of two fucans (Alves *et al.*, 1998). Any individual of this species produced only one of the two compounds. A similar phenomenon is seen in the species *S. droebachiensis* (Vilela-Silva *et al.*, 2002).

A regular fucoidan fraction from *Ascophyllum nodosum*

An anticoagulant fraction isolated from *A. nodosum* fucoidan by acid hydrolysis and centrifugal partition chromatography (fraction $H^3$5,p) (Chevolot *et al.*, 1999) gave NMR spectra which at first appeared heterogeneous. Two residue types were clearly identified as present: 3-linked, 2-*O*-sulphated, and 4-linked, 2,3-*O*-disulphated fucose.
Gel permeation chromatography showed that this fraction consisted of oligosaccharides, mostly 8-14 residues in length. The apparent heterogeneity was due to end residues: the oligosaccharides had a regular repeating disaccharide structure. 2D nuclear Overhauser effect spectroscopy in the rotating frame (ROESY) is a common and powerful technique for determination of the sequence and conformation of oligosaccharides, and in this case showed definitively that the two residue types previously found were present in the fucoidan fraction $H^3$5,p in strict alternation (Chevolot *et al.*, 2001). This pattern, though very regular, differs from that found in echinoderm fucans, as it contains two alternating linkage positions.
The NMR spectra of whole fucoidan from *A. nodosum*, and of *Fucus vesiculosus*, are similar and both are complex due to heterogeneity. However, signals corresponding to those of the two residue types in the oligosaccharide clearly make up most of the intensity present in the spectrum of fucoidan from both species (though the NMR spectrum of fucoidan from *Laminaria brasiliensis* is different (Mulloy *et al.*, 1994) At around 10 monosaccharide units in length, the oligosaccharides which comprise fraction $H^3$5,p are too long to be typical side-chains, and may therefore represent a backbone structure for the *A. nodosum* fucoidan, with heterogeneity provided by sidechains made up of sulphated fucose and other sugars such as galactose and glucuronic acid. This alternation of 3- and 4-linked fucose has also been identified in fucoidan from *Fucus evanescens* (Bilan *et al.*, 2002). The presence of fucoidans with uniformly 3-linked backbones has been demonstrated for some species (Chizhov *et al.*, 1999; Nishino *et al.*, 1991) and some 3- linked fucan may be present in *A. nodosum* fucoidan (Marais & Joseleau, 2001).

Mechanisms of fucoidan activity

Fucoidan activities are often compared with those of the mammalian sulphated polysaccharide heparin, a highly sulphated form of heparan sulphate. However not all fucoidan activities can be explained in this way; for example the endogenous ligands for selectins and the macrophage scavenger receptor are not glycosaminoglycan structures.
It must be admitted that sulphated polysaccharides may interact with proteins non-specifically, through high density of negative charge, but it is also the case that these polysaccharides may also act with a high degree of specificity, dependent largely on the geometrical arrangement in space of sulphate substituents; the sea urchin egg-jelly fucans demonstrate

this. The trisulphated disaccharide repeating unit of fucoidan from *A. nodosum* may be reminiscent of the main repeating unit of heparin (fig. 2) but the biological activities of fucoidan may not all arise from the same structural features. The overwhelming majority of biological studies make use of relatively crude preparations available commercially, but some recent studies of purified fucoidans and fucoidan fractions can shed some light on the structure-function relationships.

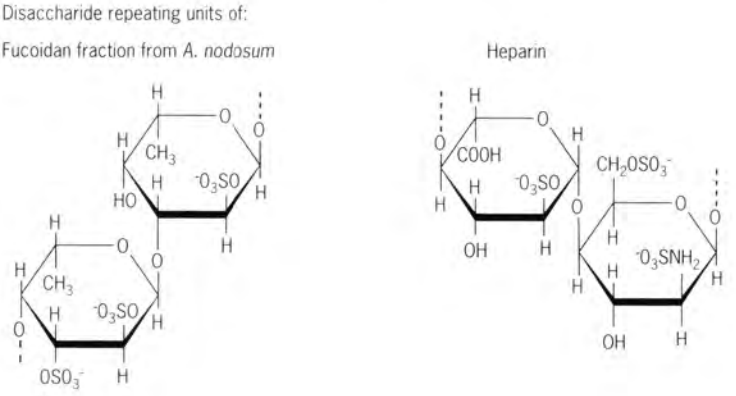

Figure 2
Both the oligosaccharide $H^3$5,p from *A. nodosum* fucoidan, and the mammalian glycosaminoglycan heparin, contain a high proportion of a trisulphated disaccharide repeat unit. The saccharide backbone structures are however very different. In the fucoidan fraction, 3- and 4-linked fucose residues alternate, whereas in heparin 4-linked α-L-iduronic acid and 4-linked α-D-glucosamine alternate.

Anticoagulant activity of low molecular weight fucoidan fractions was found to depend not only on molecular weight and sulphate content, but more precisely on 2-*O*-sulphation and 2,3-*O*-disulphation levels (Chevolot *et al.*, 1999). A separate study compared the anticoagulant activity of fucoidan fractions, some of which had been partially desulphated. For these fractions, in which the pattern of sulphation had been disrupted, activity was strongly correlated with sulphation and molecular weight. It was notable that most of the samples in which the native sulphation patterns were intact had higher activity (both as anticoagulants *in vitro* and, markedly, as antithrombotics *in vivo*) than would be predicted from their sulphation and molecular weight (Boisson-Vidal *et al.*, 2000).

Proliferation of smooth muscle cells is inhibited by both heparin and fucoidan (Patel *et al.*, 2002) and the activities of the two sulphated polysaccharides are distinguishable. Fucoidan is at least 400 times more potent than heparin, and can inhibit serum-induced mitogenesis in heparin resistant smooth muscle cells. An interesting observation was that crude commercially available fucoidan was more potent than purified fucoidan; some highly potent species, possibly another sulphated polysaccharide, has been discarded in the purification.

Although fucoidans are well-known to be ligands for selectins and macrophage scavenger receptors, they are also capable of interacting directly with cytokines such as interleukins (Ramsden & Rider, 1992).

Several fucoidan preparations are capable of inhibiting interferon-α induced endothelial cell immunomodulation more effectively than heparin (Sharma *et al.*, 1999) and in this case pure fucoidan was almost twice as active as the crude material.

Conclusion

These few instances indicate that fucoidans present a plentiful source of sulphated structures which could be developed to exhibit specific biological activities with therapeutic potential; not only as antithrombotic agents but for immunomodulatory and antiproliferative properties. The multiplicity of these different biological activities, however, argues against the likelihood of whole fucoidan, or of incompletely characterised fucoidan fractions, as candidates for therapeutic use. Manufacturers of pharmaceuticals are more likely to be interested in preparations with well-characterised structure, for which one characteristic activity predominates. The example of heparin may be instructive here. It is possible to develop synthetic drugs based on particular structures with, for example, antithrombin-binding properties (Bauer, 2001). On the other hand, it is also possible to make useful drugs from oligosaccharide mixtures (for example low molecular weight heparin), because the level of characterisation of such mixtures, both in terms of structure and function, is high. There is no reason in principle why the fucoidans of brown algae should not yield equally useful therapeutic products, but to achieve this aim it will be necessary to improve our understanding of structure/function relationships. In order to correlate biological activities with particular structural features in complex fucoidans, homogeneous fucan preparations, whether native fucans or carefully prepared fragments of fucoidans, will be invaluable research reagents.

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Antithrombotic and proangiogenic effects of sulphated polysaccharides from marine origin

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Introduction

Atherosclerosis and thrombosis are the most frequent causes of morbidity and mortality in the industrialized world. Blood stasis which induces activation of the coagulation cascade is strongly involved in venous thrombosis onset. Arterial thrombus formation is usually secondary to atherosclerotic plaque disruption, which results from exposure of highly thrombotic proteins, such as tissue factor, to the flowing blood. Atherosclerotic lesions correspond to the accumulation in the vessel intima of macrophages, lipids and smooth muscle cells.
The endothelial cells, which are the cell type lining the healthy blood vessels, are involved in many physiological functions, including protection against thrombosis. When atherosclerotic plaque disruption occurs, the loss of endothelial integrity led to platelets deposition and activation of blood coagulation, triggering arterial thrombus formation. Downstream the thrombus, ischaemic areas are formed which will become necrotic if no neovascularization occurs. Unfractionated heparin (UH) or low molecular weight heparin (LMWH) are sulphated polysaccharides from mammalian origin. They are the most widely used antithrombotic drugs. They are very effective against venous thrombosis but not against arterial thrombosis in which vessel wound repair and neovascularization of ischaemic areas are required. Moreover, in this last indication, these drugs have to be used at very high doses corresponding to a high anticoagulant effect and thus to a high haemorrhagic risk, related to their narrow efficacy to safety ratio. The relative ineffectiveness of heparins and their side effects have prompted researchers to study new antithrombotic agents and, among them, new polysaccharides. Fucoidans are polysaccharides extracted from algae, and their marine origin excludes theoritically any risk of contamination with non-conventional agents.

Fucoidan fractions

Fucoidans are primarily composed of α (1,2) or α (1,3)-L-fucose ($4.0S0_3$) with branching (D-xylose, D-galactose, D-mannose, D-glucuronic acid) or sulphate at position 3 or 2. (Mauray *et al.*, 1995). They were fractionated in order to obtain fractions with molecular weights (MW: 20 000 Da and 7 000 Da) comparable with those of UH (MW: 15 000 Da from Sanofi) and a LMWH (Dalteparin, MW: 5 000 Da from Kabi Pharmacia, St-Quentin, France). These molecular weights are compatible with *in vivo* experiments and possible clinical use.

Anticoagulant activities of the fucoidan fractions

The studied fucoidan fractions exert their anticoagulant activity by enhancing thrombin (the main enzyme of the coagulation cascade) inhibition mainly via heparin cofactor II but also via antithrombin. They have no anti-factor Xa activity. They are less anticoagulant than heparin; for example, *in vitro*, when tested by activated partial thromboplastin time (APTT) and thrombin time (TT), on a weight basis, a 30 to 40 times higher concentration of a 20 000 Da fucoidan fraction was required to obtain the same anticoagulant effect as UH (Mauray *et al.*, 1995). Similar results were obtained when LMW fucoidan and LMWH were compared (Millet *et al.*, 1999).

Antithrombotic activities of the fucoidan fractions

The venous antithrombotic effect of fucoidans, compared to heparin, was tested in a rabbit model of venous stasis (Wessler model) with bovine factor Xa as thrombogenic stimulus.

We have first shown that the 20 000 Da fucoidan fraction had venous antithrombotic activity following intravenous injection to rabbits. A 20 - fold higher amount of fucoidan than UH was required to obtain 80% thrombus inhibition, a ratio lower than that of the anticoagulant effect measured by *in vitro* clotting assays. The anticoagulant effect is related to an haemorrhagic risk (Mauray *et al.*, 1995). Fucoidans are 30 to 40 times less anticoagulant, on a weight basis, than unfractionated heparin (UH); LMWH are less anticoagulant than UH, and LMW fucoidan less than the 20 000 Da fucoidan fraction. We thus compared, in the same rabbit model, the venous antithrombotic activity of a LMWH fucoidan and a LMWH used by subcutaneous route. The LMW fucoidan exhibited a dose-related venous antithrombotic activity. At the same antithrombotic activity, LMWH fucoidan exhibited a lower effect on *ex vivo* coagulation tests, and a lower prolongation of the bleeding time than the LMWH, which corresponded to a weaker haemorrhagic effect (Millet *et al.*, 1999) (fig. 1).

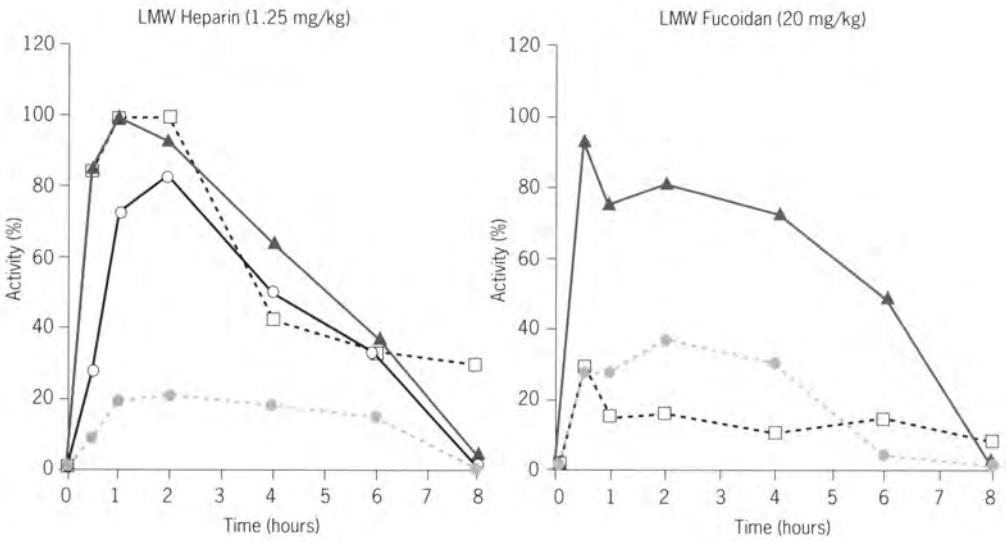

Figure 1
Time-course of *ex vivo* anticoagulant and antithrombotic activites of LMW fucoidan at 20 mg/kg (ED_{80}) and LMW Heparin at 1.25 mg/kg (dose close to the ED_{80}). The compounds were administered by subcutaneous route at various times before thrombus induction by bovine factor Xa, in the rabbit. Blood was collected just after thrombosis induction for *ex vivo* assays : TT (□) ; APTT (●); antithrombotic activity (▲) and, for LMW Heparin, anti-Xa activity (○). Each result is the mean of experiments performed on groups of 5-7 treated and control animals.

We have then shown that LMW fucoidan exhibited arterial antithrombotic properties in rabbit and rat at the same doses than in a venous thrombosis model. In the same arterial thrombosis models, LMWH and UH have to be used at much higher doses than in the venous thrombosis model. Thus, the anticoagulant effect, the prolongation of the bleeding time and the haemorrhagic risk are much more pronounced, in this arterial thrombosis model, with heparins than with fucoidan (Colliec-Jouault *et al.*, 2001).

Fucoidan-endothelial cell interactions

Endothelial wound repair is a crucial step to prevent rethrombosis and restenosis of a damaged arterial vessel wall. Heparin binding growth factors such as fibroblast growth factor-1 and -2 (FGF-1 and FGF-2) are able to modulate endothelial cell proliferation, migration and differentiation. As a consequence of their structure, rich in sulphate and carboxylic groups, we hypothezised that fucoidans could bind to these growth factors; we thus studied the fucoidan-endothelial cell interactions in the absence or in the presence of FGF-1 or FGF-2.

In the absence of growth factor, fucoidan (MW 20 000 Da) stimulated the proliferation of human umbilical vein endothelial cells (HUVEC) in culture, whereas UH inhibited it. In the presence of FGF-1, both fucoidan and UH potentiated endothelial cell proliferation and migration. Fucoidan modulated FGF-2 induced cell proliferation, in a way depending on FGF-2 concentration, whereas UH had an inhibitory effect (Giraux *et al.*, 1998a,b). Moreover we demonstrated that fucoidan, as heparin and at the same doses, (thus at a lower anticoagulant activity for fucoidan) induced free and total tissue factor pathway inhibitor (TFPI) release from HUVEC (fig. 2). TFPI regulates the tissue factor-dependent pathway of

blood coagulation and its release from the endothelium by heparin is claimed to contribute to the heparin antithrombotic effect. Thus our data suggest that TFPI released from vascular endothelial cells may contribute to the antithrombotic effect of fucoidan (Giraux *et al.*, 1998a,b).

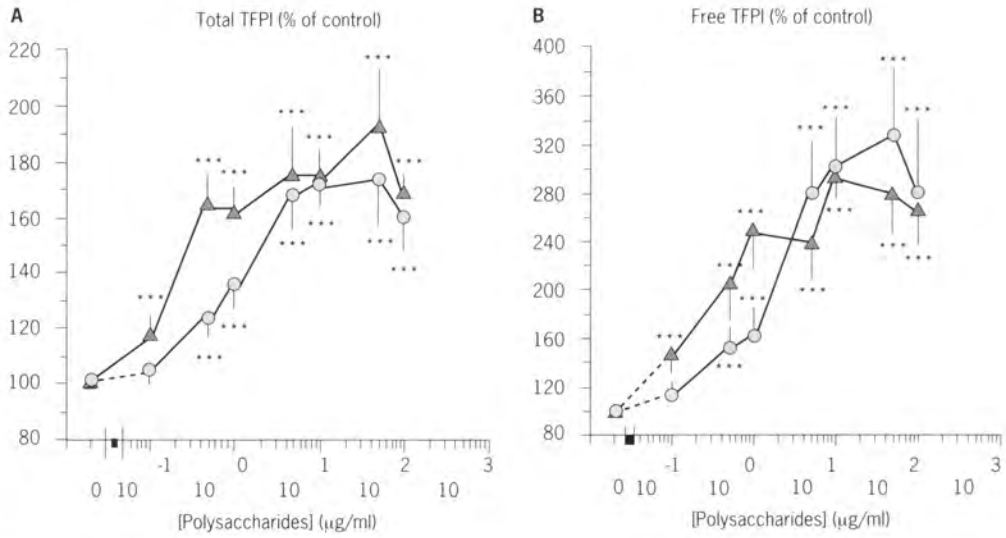

Figure 2
Concentration-dependent effect of fucoidan and heparin on TFPI release from HUVEC.
Confluent endothelial cells were incubated for 1 h with culture medium supplemented with increasing concentrations of fucoidan (▲) or heparin (●). Total (A) and free (B) TFPI antigen levels were measured in the supernatant by ELISA.

Fucoidan induces angiogenesis *in vitro*

Angiogenesis corresponds to the formation of new vessels, for example in ischaemic areas after arterial thrombosis. Endothelial cell differentiation is a crucial step of angiogenesis. After vessel injury, FGF-2 is secreted. It is known to induce neovascularization in several experimental models. This effect is mediated by FGF-2 interaction with both high- and low-affinity cell surface receptors, the last one being heparan sulphate proteoglycans (HSPG). The FGF-2-receptor interactions induce intracellular signaling activity responsible for cell proliferation, migration and differentiation. The cell differentiation could be characterized by the expression, at the cell surface, of proteins such as AVB3 integrin or β1 and α6 integrin subunits. These integrins are adhesion receptors mediating the attachment of endothelial cells to the basal membrane. Their expression will allow the cells which have migrated to form new vessels by adhesion to the newly formed extracellular matrix. We have hypothesized that fucoidans are able to modulate endothelial cell differentiation induced by FGF-2. We have thus studied the ability of HUVEC to form vascular tubes on a reconstituted basal membrane, Matrigel, which is a well-known *in vitro* angiogenesis model. The experiments were performed in the presence or absence of sulphated polysaccharides (fucoidan, MW: 20 000 Da, or UH), and in the presence or absence of FGF-2; on the same batch of cells, the expression of α6 and β1 integrin subunits was quantified by flow cytometry.

In the absence of FGF-2, endothelial cells were unable to form vascular tubes on Matrigel. In the presence of FGF-2 with no addition of polysaccharides, the cells were differentiated in a partially organized vascular network. When fucoidan was added to FGF-2 at concentrations which are antithrombotic in a rabbit model, the density of the network was increased and the tubes organized in closed areas, whereas UH had no effect (fig. 3). The tube formation on Matrigel was related to an overexpression of $\alpha 6$ subunit integrin, which was highly significant in the presence of FGF-2 and fucoidan. An anti-FGF2 antibody markedly reduced the a6 overexpression, which indicated that fucoidan acts mainly on vascular tube formation by potentiating the effect of FGF-2 on 6 expression (Matou *et al.*, 2002)

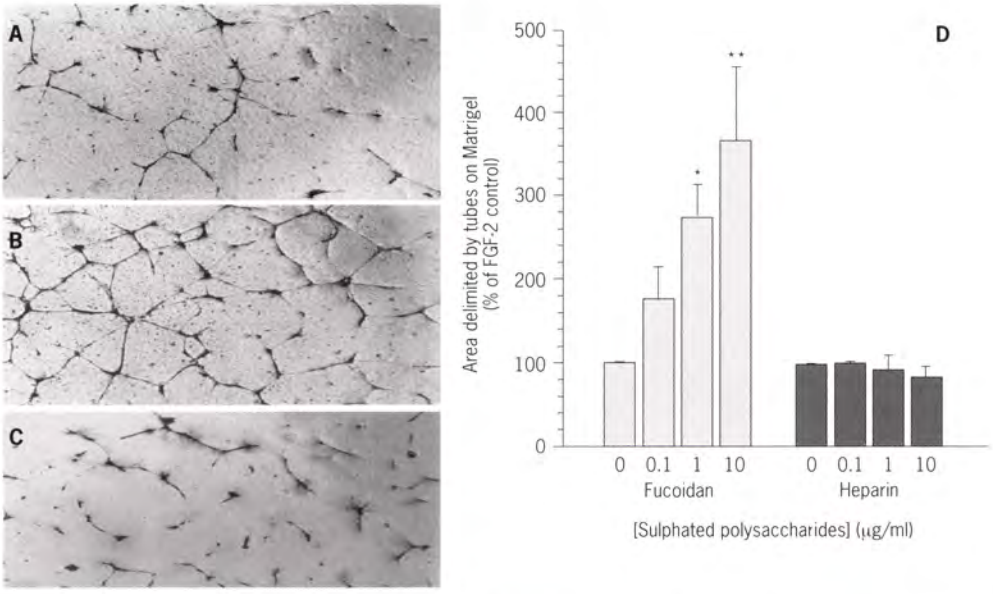

Figure 3 - Effect of fucoidan and heparin on FGF-2-induced tube formation on Matrigel.
Photographs present vascular tube formation on Matrigel from huvec previously treated with FGF-2 alone (A), FGF-2 plus fucoidan at 1µg/ml (B) or heparin at 1µg/ml (C). For different concentrations of fucoidan (grey bars) and heparin (black bars), in the whole surface of each well, the total enclosed area delimited by the tubes was measured with the Biocom image analysis sytem (D). Values are expressed in percentages, 100% corresponding to huvec treated with FGF-2 alone. Results are means ± SE of six determinations for fucoidan and four for heparin. (*) and (**) significantly ($p < 0.05$ and $p > 0.01$) different from FGF-2 alone.

Conclusion

Fucoidans are polysaccharides with high antithrombotic activities, low anticoagulant effect and thus low haemorrhagic risk in rabbit. They inhibit smooth muscle cell proliferation (Logeart *et al.*, 1997) which is a crucial step in the formation of atherosclerotic lesions, and exhibit an

in vitro proangiogenic effect. They are thus potential promising antithrombotic drugs which could be of interest in preventing restenosis or potentiating neovascularization of ischaemic areas.

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Anticomplementary activity of the marine polysaccharide fucoidan: determination of the targeted proteins and of their interactions

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The fucoidan extracted from brown seaweed like *Ascophyllum nodosum* is a sulphated fucose-based polysaccharide whose structure remains unclear, while it exhibits important biological properties, such as anti-complementary activity.

The complement is an important biological system involved in the host defence against infection. About 20 proteins are involved in the complement activation through an enzymatic reactions cascade along two pathways, classical and alternative that both lead to the formation of the anaphylatoxins and to the membrane attack complex responsible for the destruction of the targeted cell (fig. 1). But, in some cases, this defence system is activated beyond any control and may cause serious damages to the host himself (i.e. anaphylactic shock, various neurodegenerative diseases, skin bullous pathology).

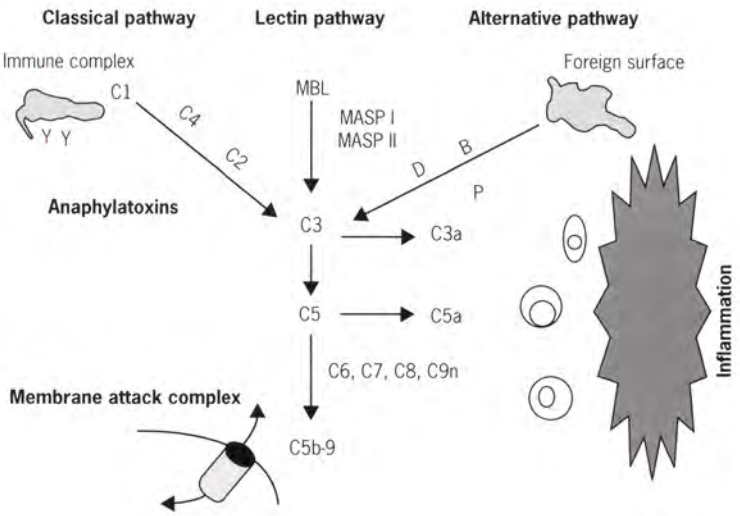

Figure 1
Scheme of the different ways of activation of the complement system.

We worked with two different fractions of fucoidan named F and P (8,500 g/mol and 30% sulphate and 3,000 g/mol and 30% sulphate, respectively) whose structure has been recently proposed (fig. 2) (Chevolot *et al.*, 1999; Daniel *et al.*, 2001). We have showed that both fucoidans were able to block efficiently the classical pathway of activation, (IC50 9 µg/ml and 16 µg/ml for fucoidans F and P respectively) and as well as the alternative one (IC50 6 µg/ml for fucoidan F).

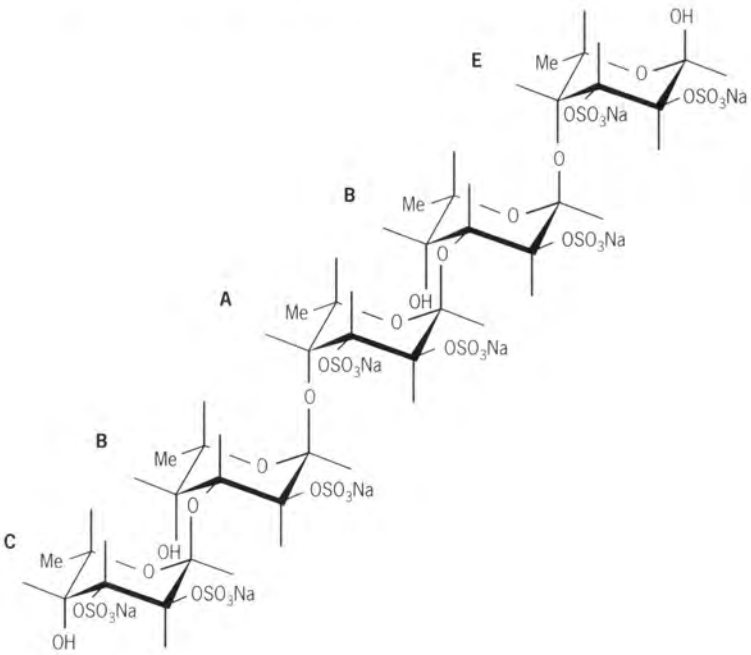

Figure 2
Proposed structure of fucoidans F and P from the brown algae *Ascophyllum nodosum* (Chevolot *et al.*, 1999).

Our goals are to identify which proteins of the cascade are specifically targeted by fucoidan and to characterize their interactions with the polysaccharide. As regard the classical pathway we showed that fucoidan mainly interfere at the first steps of the activation, by inhibiting the formation of the complex C1 (3 µg of fucoidan F lead to a 60% inhibition of the complex formation) and of the classical C3 convertase. C1 is an enzymatic complex responsible for the recognition of non-host cells that triggers the activation of the classical pathway. The classical C3 convertase, the second main enzymatic complex, is at a limiting step in the cascade before the amplification loops of the activation. We demonstrated by haemolytic assays that fucoidan interacts with components of C1 and C3 convertase, the proteins C1q and C4 respectively, and that it has no action on their enzymatic components, the serine proteases C1r, C1s or C2. Indeed, pre-incubation of C1q and C4 with increasing concentrations of fucoidan resulted in an increased inhibition of the classical pathway (fig. 3). Specific labelling of lysine residues of C1q resulted in the inhibition of its haemolytic activity. The

presence of fucoidan during the labelling reaction preserved the functional activity of C1q, likely through the protection of essential lysines. Hence, we assume that fucoidan interacts with the essential lysines residues of C1q in such a manner that its functionality is impaired resulting in the inhibition of the classical pathway activation. Furthermore, we confirmed these results by co-affinity electrophoresis on agarose gel analyses that showed binding of fucoidan to C1q. This inhibition of the Complement cascade at the C1 step is amplified by an additional action of fucoidan on the C4 protein. This latter has been investigated by capillary electrophoresis analysis.

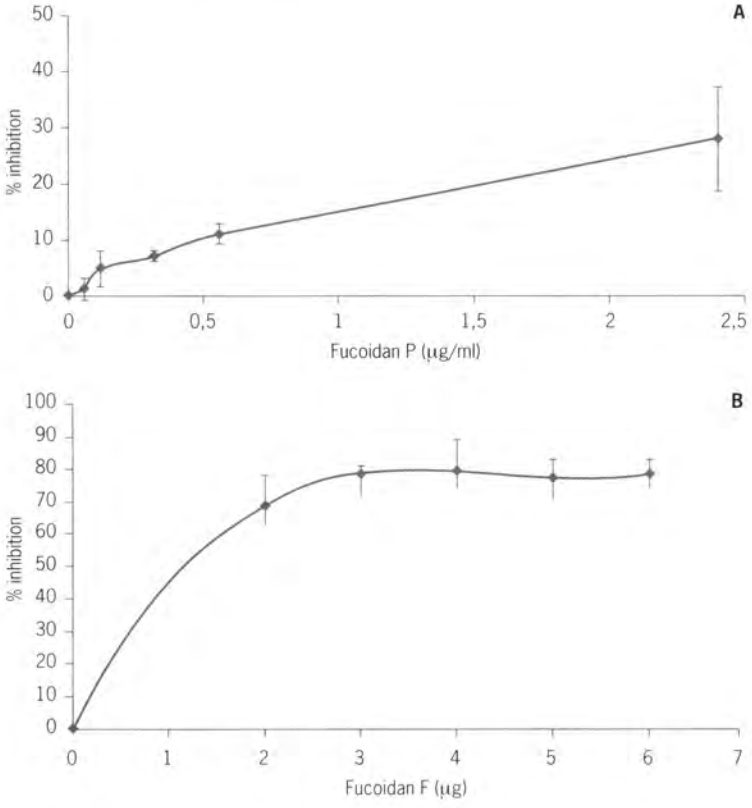

Figure 3
Inhibition of the haemolytic activity of C1q by fucoidan P (A) and of the haemolytic activity of C4 by fucoidan F (B).

Concerning the alternative pathway, we have shown know that fucoidan F blocks the consumption of the Factor B (50 µg/ml lead to 50% inhibition), which is involved in the formation of the alternative C3 convertase. During this formation, fucoidan F does not inhibit the cleavage of B by the enzyme Factor D. Preliminary experiments indicated that this fucoidan could interfere with the association of the resulting B fragment (Bb) to the protein C3b so that the formation of the alter-

native C3 convertase (C3b Bb) bound to the cell membrane could be hampered. It is also interesting to underline that fucoidan P has no such effect on the formation of the alternative C3 convertase.
The other part of our work concerned the polysaccharide itself since we are now trying to identify the structural requirements necessary to the anti-complementary effect. We especially focus our research on the positions of the sulphate groups on one hand and on the rate and the position of the branching on the other hand.

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Production of fucoidanase from marine fungus *Dendryphiella arenaria* TM94 by solid substrate fermentation using marine algae resources

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Abstract

A marine fungus strain TM94 isolated from sea sand was identified as *Dendryphiella arenaria*. It could produce fucoidanase by solid state fermentation using marine algae. The conditions for producing the enzyme were studied. The result showed that three marine substrates such as *Laminaria* sp. and *Fucus vesiculosus* and crude fucoidan could promote the fucoidanase production. Among three materials, crude fucoidan was the best substrate for *Dendryphiella arenaria* TM94 to produce the enzyme. The highest fucoidanase activities reached 32.75 IU per gramme of medium after cultured 24 hours. The optimal temperature for fucoidanase yielding was 28°C. The best inoculum level for enzyme production by solid state culture was 2 ml spore suspension ($1.0x10^7$ spore/ml). The properties of enzyme were also studied. The result showed that the enzyme exhibited a maximal activity at 50°C and at pH 4.6. The enzyme showed relatively stable below 40°C and between pH 5.7~6.2. The enzyme could be applied in hydrolysis fucoidan.

Introduction

Fucoidan, a kind of heteropolysaccharides, is mainly composed of sulphated L-fucose and a small proportion of galactose, mannose, xylose and uronic acids (Kloareg & Quatrano 1980). It mainly exists on the surface of sea brown alga, mixing with alginate and laminaria. The fucoidan content of brown alga varies with the change of climate and species. In China, *Laminaria* sp. produced annually are in abundance. It is important to develop these algae by-products.

In recent years, many scientists have paid attention to the relationship between preparation and biological activities of fucoidan. The investigated reports showed that fucoidan, especially low molecule weight

fucan (LMWF), had its clinic activities (Chevolot *et al.*, 1999; Duerig *et al.*, 1997; Grauffel *et al.*, 1989; Colliec-Jouault *et al.*, 1991; Millet *et al.*, 1999; Blondin *et al.*, 1996):

- (1) antivirus, some researchers reported that the sulphated fucoidan extracted from *Laminaria* sp. has its antivirus effects on HIV as well as various DNA and RNA virus. The result showed that fucoidan could slow down the infection of HIV into white cell (Lynch *et al.*, 1994; Baba *et al.*, 1998);
- (2) anticoagulant and antithrombic activity. The most recently published papers indicated that such activities associated with the low molecular weight fucans (Chevolot *et al.*, 1999; Mauray *et al.*, 1998). The results showed that the anticoagulant activity was related to molecular weight, and the sulphating level as well;
- (3) other activities. Fucoidan could be used as drugs for antitumoral (Ellouali *et al.*, 1993), antifertilizing and plasminogen (Nishino *et al.*, 2000).

As fucoidan molecular weight is too large for use in drugs, low molecular weight fucans were prepared. There are some methods such as chemical acid hydrolysis, physical radical cleavage (Chevolot *et al.*, 1999) and biological enzyme hydrolysis for preparing low molecular weight fucans. Among them, biological method may be the best one. Fucoidanase can hydrolyze fucoidan to small fragment without removal of side substitute groups because of its substrate hydrolysis property. There are few papers concerning fucoidanase production. Fucoidanase could be obtained from hepatopancreas of animal (Kitumura *et al.*, 1992; Thanassi & Nakada, 1967) and some bacteria such as *Pseudomonas atlantica*, *Pseudomonas carrageenovora* (Yaphe & Morgan, 1959) and *Vibrio* sp. N-5 (Furukawa *et al.*, 1992). We found a species marine fungus *Dendryphiella arenaria* TM94 could produce fucoidanase by solid state fermentation. In this paper, we described the production and properties of fucoidanase by *Dendryphiella arenaria* TM94.

Material and methods

Microorganism

Marine fungus strain TM94, isolated from the sea sand of the beach of Baltic sea near Copenhague beach, was identified as *Dendryphiella arenaria*. It was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured every 2 months. The spores were harvested after 10 days at 28°C before use.

Media and cultivation

The medium for enzyme production were wheat bran 7.5 g, glucose 0.5 g and various amount of different kinds of additive materials powder such as *Laminaria* sp., *Fucus vesiculosus* and crude fucoidan, and 6 ml seawater (containing $NaNO_3$ 4.0 g/l) in 250 ml Erlenmeyer flasks. The spore suspension 3 ml (1.0×10^7 spores/ml) was inoculated into it, then cultured at the temperature 28°C. The basal medium with none additive was used as control kind.

Preparation of fucoidan

Fucoidan was prepared from *Laminaria* sp. by method of Wu (Wu *et al.*, 2001). The crude fucoidan was extracted from *Laminaria* sp. with hot water at 80°C for 10 h, then was treated with alcohol fractionation, and the precipitate was obtained and dried. This fucoidan was used as carbon source for the cultivation of fungus.

Preparation of Fucoidanase

After fermentation, 50 ml of distilled water were added into flask containing cultured media, and the medium was homogenized and extracted for 1 h, then centrifuged. The supernatant was used as a crude enzyme solution.

Fucoidanase activity assay

Fucoidanase activity was assayed by a colorimetrical analysis of reducing sugar released from fucoidan (Furukawa *et al.*, 1992). The reducing sugar released was measured by Miller method (Miller, 1959). A mixture consisting of substrate solution 1% fucoidan (*Fucus vesiculosus*, purchased from Sigma) and enzyme solution 0.1 ml was incubated at 50°C for 10 minutes. One unit (IU) of fucoidanase activity is defined as the amount of enzyme that releases 1μmol of fucose per minute under the assay conditions.

Effect of pH on fucoidanase activity

The optimal pH of fucoidanase activity was determined by measuring the enzyme activity at 50°C at various pH levels between 4 and 7.5. The pH stability of the enzyme was determined by assaying the residual activity under standard conditions after incubating enzyme for 24 h at 30°C at various pH levels ranging from 4 to 9. The buffer was phosphate solution.

Effect of temperature on fucoidanase activity

The optimal temperature of fucoidanase activity was determined by measuring enzyme activity at pH 5.5 at temperature from 30°C to 70°C. The thermostability of the enzyme was determined by assaying the residual activity under standard enzyme assaying conditions after incubating the enzyme solution for 1h at various temperatures from 30°C to 70°C.

Results and discussion

Component of the solid state fermentation

Several kinds of materials such as wheat bran, corncob and rice straw were selected as medium to produce the enzyme by *D. arenaria* TM94. Table 1 showed the result of the effect of these media and their complex with glucose on the enzyme production. Marine substrates such as crude fucoidan, *Fucus vesiculosus* and *Laminaria* sp. contained in the

medium were beneficial to the fungus to produce fucoidanase. The activities of enzyme reached 24.6, 18.6 and 20.5 IU/g dry medium respectively.

Table 1 - Component of the solid state fermentation.

Carbon sources	Fucoidanase activity (IU/g dry medium)
Wheat bran 7.5g	3.9
Corncob 7.5g	1.0
Straw 7.5g	1.5
Wheat bran 7.5g + glucose 0.5g	1.5
Corncob 7.5g + glucose 0.5g	2.0
Straw 7.5g + glucose 0.5g	4.4
Straw 7.5g + glucose 0.5g + crude fucoidan 0.3g	24.6
Straw 7.5g + glucose 0.5g + *Fucus vesiculosus* 0.3g	18.6
Straw 7.5g + glucose 0.5g + *Laminaria* sp. 0.3g	20.5

Effect of nitrogen sources on the enzyme production

Table 2 showed the effect of nitrogen sources on the enzyme production. Several kinds of nitrogen sources were added into medium for *Dendryphiella arenaria* TM94 to produce the fucoidanase. The result showed that among the nitrogen sources, sodium nitrate with the concentration 4g/l in the medium were the best for the enzyme production.

Table 2 - Effect of nitrogen sources on the enzyme production.

Nitrogen sources	g/l	Relative activity
CK		100
$NaNO_3$	3	149
	4	249
$(NH_4)_2SO_4$	3	217
	4	183
Urea	3	183
	4	166
Peptone	3	100
	4	217
Yeast extract	3	134
	4	52
NH_4NO_3	3	166
	4	217

Effect of various additives on the production of fucoidanase

According to the result of table 1, the marine algae materials used as additives could promote the enzyme production by *Dendryphiella arenaria* TM94. Figure 1 showed the fucoidanase production after 24 h of culture by with various substrates in media. The best additive weight into medium were 0.6 g for *Laminaria* sp. and 0.9 g for *Fucus vesiculosus* in the 8.0 g medium. The enzyme activities were 22.5 IU and 28.75 IU per gramme of medium respectively.

Figure1
Effect of additive substrate on the production of fucoidanase by *Dendryphiella arenaria* TM94.

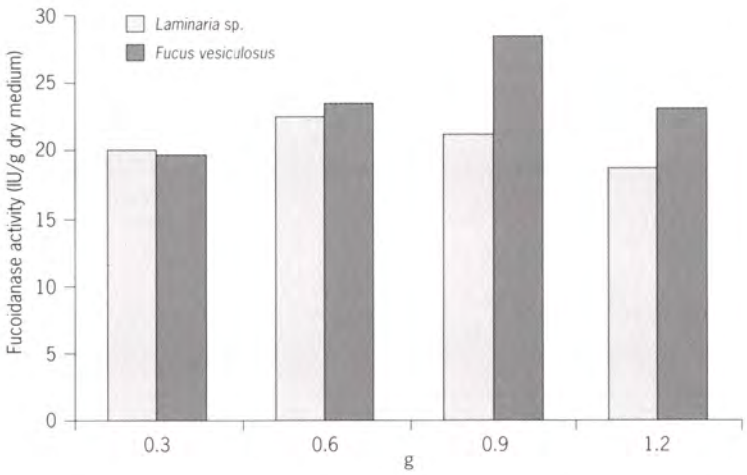
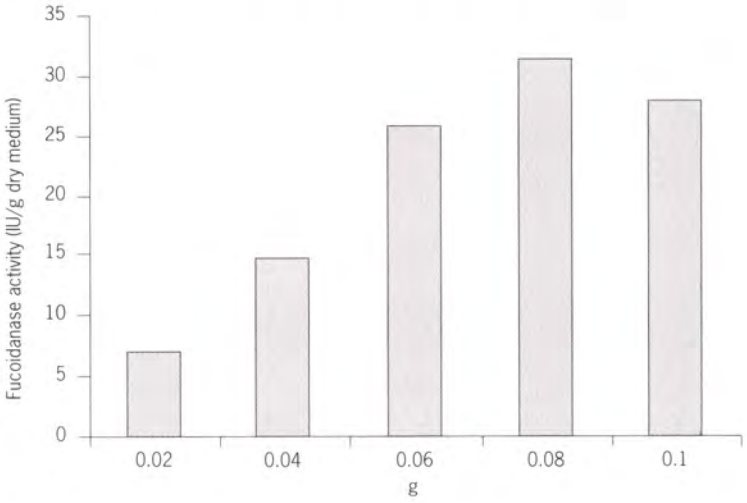


Figure 2 showed the effect of crude fucoidan as additive substrate in the media. By changing the crude fucoidan weight range from 0.02 to 0.08 g, the activity in the culture increased. The best result was adding 0.08 g substrate into medium. The enzyme activity reached 32.75 IU per gramme of medium in the test. But with the increasing of the crude fucoidan content in the medium, the fucoidanase production decreased in the test.

Figure2
Effect of crude fucoidan on the production of fucoidanase by *Dendryphiella arenaria* TM94.



Effect of temperature on the fucoidanase production

The temperature range from 25 to 32°C was set for the test. Figure 3 showed the result of temperature effect on *Dendryphiella arenaria* TM94 fucoidanase production. The optimum temperature for enzyme production was at 28°C.

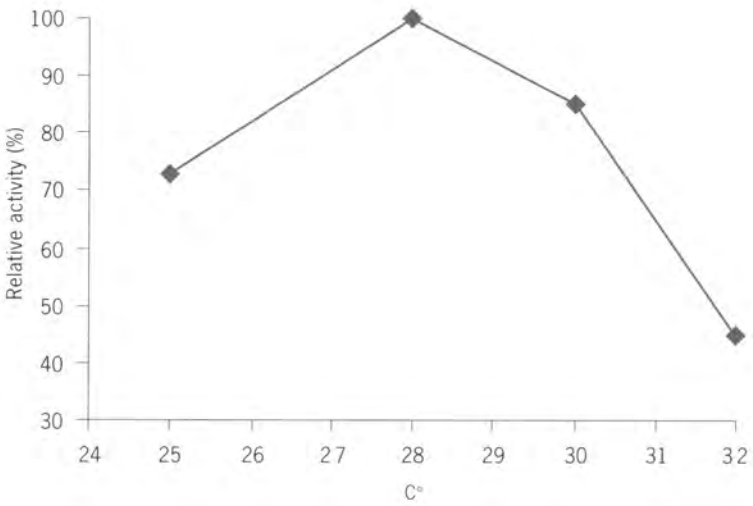


Figure 3
Effect of temperature on enzyme produced by *Dendryphiella arenaria* TM94.

Effect of inoculum level on the enzyme production

The experiment investigated that lower inoculum level would prolong incubation time of the enzyme production. On the other hand, higher inoculum level could shorten the time of the enzyme production. These both situations were not beneficial for the enzyme production. Figure 4 showed the different inoculum levels in the test. The result indicated that 3 ml (1.0×10^7 spores/ml) suspension was the best the inoculum level for the fucoidanase production.

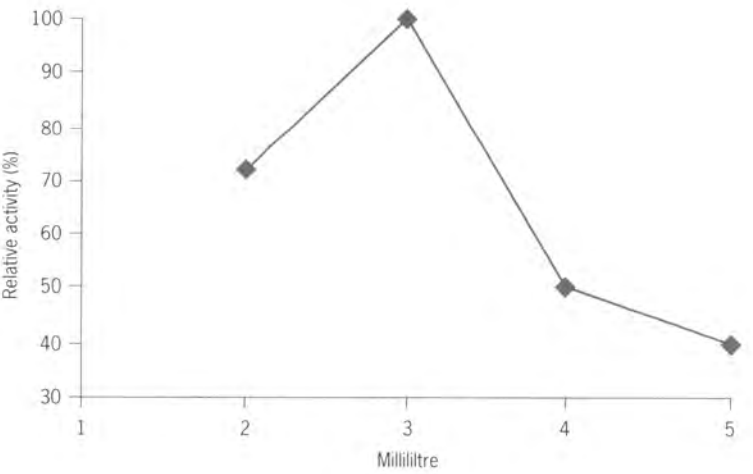


Figure 4
Effect of inoculum on enzyme produced by *Dendryphiella arenaria* TM94.

Time course of fucoidanase production

Figure 5 showed the time course of fucoidanase production by solid state fermentation in the optimal condition: fucoidanase was produced by *Dendryphiella arenaria* TM94 with the medium containing 0.08 g crude fucoidan. The enzyme activity reached 32.75 IU/g dry medium after cultured 24 hours.

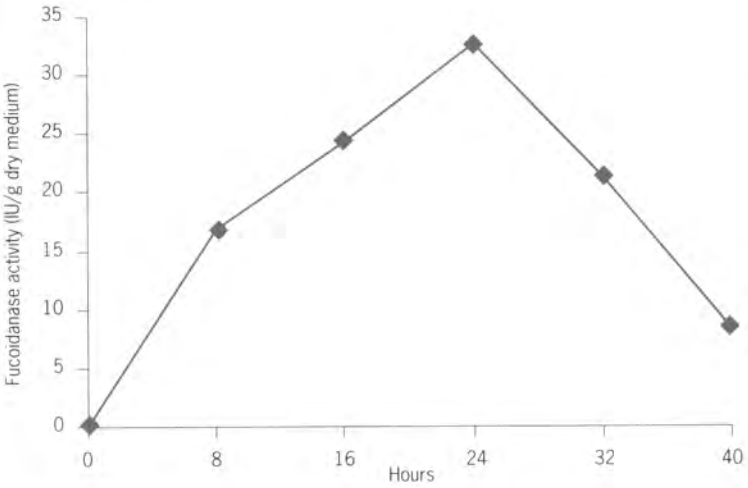


Figure 5
Time course of production of fucoidanase by *Dendryphiella arenaria* TM94.

Effect of pH on the fucoidanase activity

The effects of pH on the activity and stability of the crude fucoidanase produced from *Dendryphiella arenaria* TM94 by solid state fermentation with the medium of *Laminaria* sp. are shown in figure 6 and figure 7. The enzyme exhibited maximal activity at pH 6. The optimal pH of this enzyme is very close to a marine bacterium fucoidanase from *Vibrio* sp. N-5 (Furukawa *et al.*, 1992).

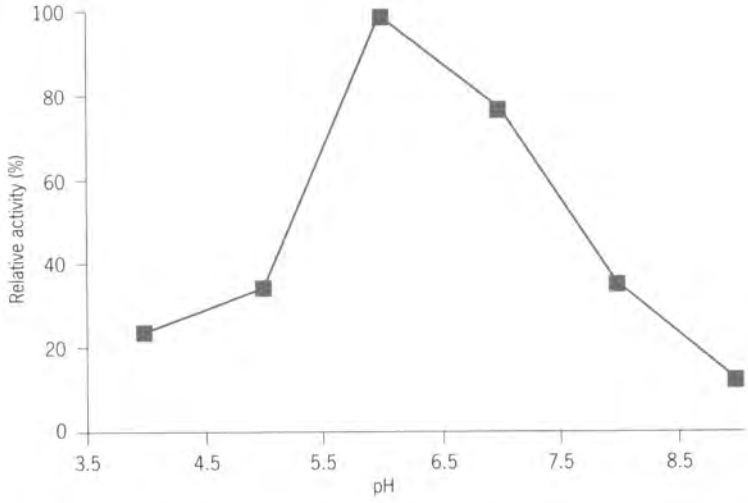


Figure 6
pH optimal of fucoidanase produced by *Dendryphiella arenaria* TM94.

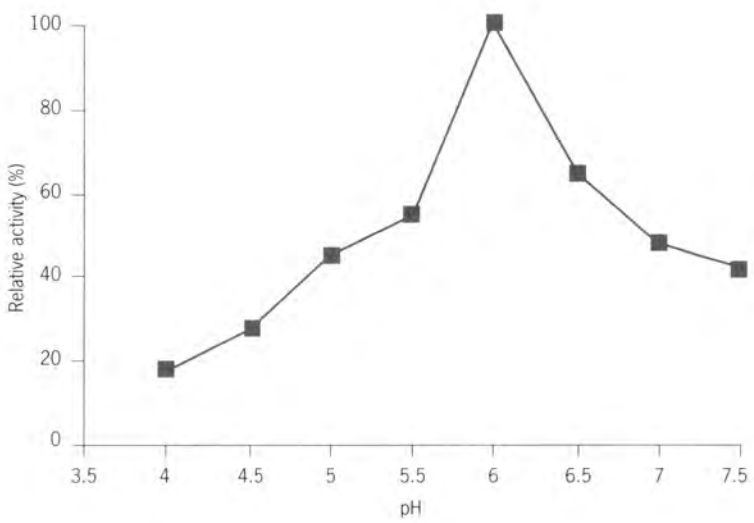


Figure 7
pH stability of fucoidanase produced by *Dendryphiella arenaria* TM94.

The enzyme showed more stable at pH 6. At pH range 5.5~7.5, the relative activity is more than 50%. The relative activity declined to 20% or below at pH 4 and pH 8.5 or above with the standard method determination was done.

Effect of temperature on fucoidanase activity

Figure 8 and figure 9 showed the effect of temperature on the activity and stability of the fucoidanase produced by *Dendryphiella arenaria* TM94 on *Laminaria* sp. The enzyme showed maximal activity at 50°C. The enzyme stability displayed that the residual enzyme activity retained about 80% of initial enzyme activity after incubated at 40°C for 1 hour.

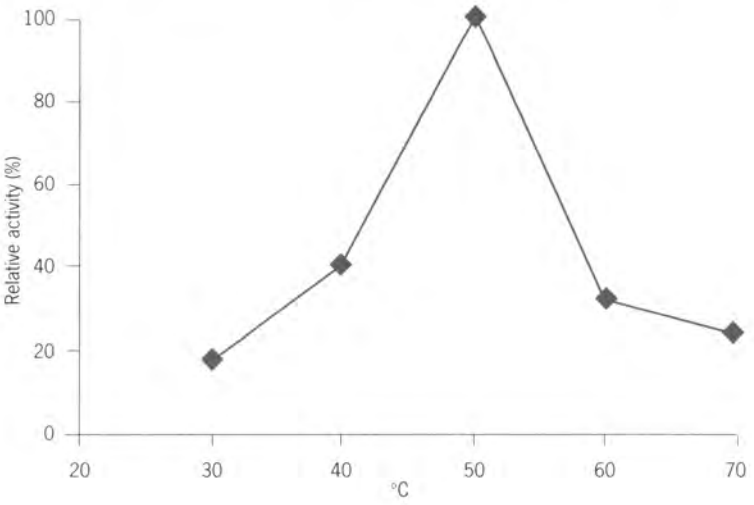


Figure 8
Temperature optimal of fucoidanase produced by *Dendryphiella arenaria* TM94.

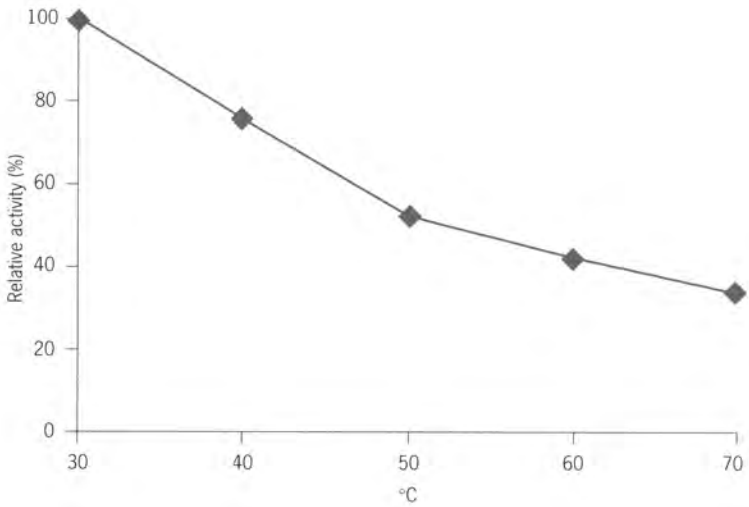


Figure 9
Temperature stability of fucoidanase produced by *Dendryphiella arenaria* TM94.

The temperature of enzyme lost half activity was 55°C. The temperature optimum of this enzyme is different from that of marine *Vibrio* sp. N-5 (Furukawa *et al.*, 1992). The enzyme of *Vibrio* sp. N-5 showed optimal temperature at 45°C. The result displayed that the fucoidanase produced by marine fungus *Dendryphiella arenaria* TM94 had higher heat tolerance than the enzyme produced by marine bacteria *Vibrio* sp. N-5.

Enzymatic hydrolysis of fucoidan

Both materials such as crude fucoidan and fucoidan (Sigma) were used as substrates. They were incubated with fucoidanase at 50°C. The release of the reducing sugar from hydrolyzing solution was monitored. The results were showed in figure10.

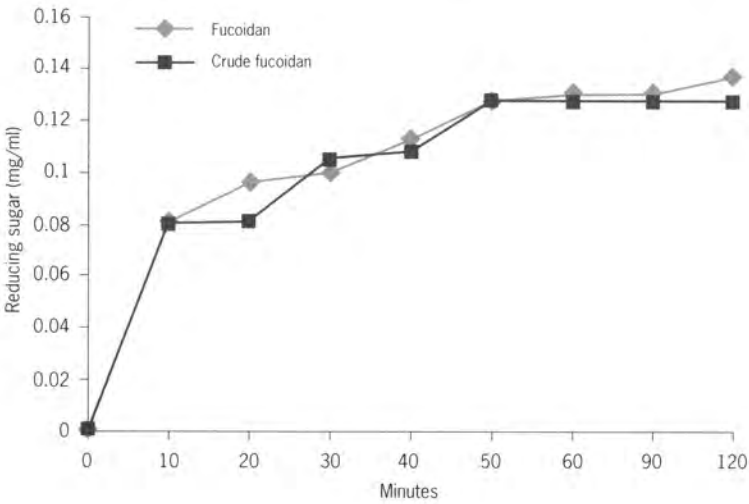


Figure 10
Time course of the enzyme hydrolyzing activity.

Conclusion

The results presented in this paper indicated that the extracellular fucoidanase of *D. arenaria* could be obtained by solid state fermentation. Like many others polysaccharide hydrolysases, fucoidanase is an induced enzyme. It was produced on the fucoidan or fucoidan containing medium. The intracellular enzyme of *Vibrio* sp. N-5 was induced by fucoidan while fucoidanase produced from *Dendryphiella arenaria* TM94 was extracellular enzyme. The result showed that fucoidan or fucoidan containing medium could promote the marine fungus to produce higher enzyme content in the medium.

Since the biological activity of fucoidan, especially low molecular weight fucan, has presented many effectual functions, the method how to get the low molecular weight fucoidan product fascinated many scientists. Since the fucoidanase has exo- (Furukawa *et al.*, 1992) and endo- type (Kitumura *et al.*, 1992; Thanassi & Nakada, 1967) hydrolysis activities and substrate specificities, it can cleave the macromolecular fucoidan to low molecular weight fucan without removal of the side chain. Maybe it is a useful way to produce the low molecular weight fucan for clinical use.

The fucoidanase produced from *Dendryphiella arenaria* TM94 had a relatively higher temperature optimal (50°C), and similar pH optimal (5.6) compared with that of marine bacteria *Vibrio* sp. N-5. The fucoidanase were stable below 40°C and pH ranging from 5.7 to 6.2. The enzyme had ability of hydrolyzing fucoidan.

This research provided a method of fucoidanase production from marine fungus by solid state fermentation using marine algae materials and the potential application in LMWF preparation.

Acknowledgements

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Fermentations using moulds hydrolysing xylan improve in *vitro* digestibility of *Palmaria palmata*, a red seaweed

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Abstract

Palmaria palmata (dulse) is an edible red alga which constitutes a potential protein source in the human diet. However, previous studies showed that the digestibility of dulse proteins seemed to be limited by the non-proteic fraction e.g. fibers. The water-soluble xylan, a polymer of xylose, which is present in high proportions in dulse, could be involved to explain the weak digestibility of the *Palmaria palmata* proteins. To limit the influence of fibers and to improve the nutritional quality of these algal proteins, we have treated *Palmaria palmata* by fermentation by moulds which is largely used in Far East and in alimentary industry.
After a 30-min predigestion by pepsin followed by a 6-hour digestion into a cell dialysis containing porcine pancreatin, the *in vitro* protein digestibility of fermented samples was 45% to 65% of that of casein while digestibilities were respectively 36% and 20% for fermentation blank and crude alga.

Introduction

Seaweeds are potentially a good source of proteins. Some are traditionnally consumed in Far East and authorized in human nutrition in western countries but remain a neglicted vegetable. In general, brown seaweeds contain a low protein level (5 to 11% of dry weight, DW). Others algae such as red seaweeds present a protein content (30-40% DW) comparable to that of leguminous such as soya bean. For example, proteins of *Porphyra tenera* represent 47% DW (Fujiwara-Arasaki *et al.*, 1984). *Palmaria palmata* or dulse, an edible red alga, is present on French Brittany and North Europe coasts. It is consumed like a vegetable or condiment. Dulse is rich in proteins, 8 to 35% DW (Morgan *et al.*, 1980). However the introduction of edible seaweeds in human feeding is reduced and limited because of the weak disponibility of protein and high proportions of polysaccharides. In the case of dulse, xylan which is a polymer of xylose, could be responsible of the weak digestibility of proteins

(Galland-Irmouli *et al.*, 1999; Bobin-Dubigeon *et al.*, 1997). The use of polysaccharidases is a method to improve the disponibility and digestibility of seaweeds protein (Fleurence *et al.*, 1995).

The fermentation process is largely used in Far East. Fermentation of soya bean by *Rhizopus microsporus* var. *chinensis* gives "tempeh" and fermentation by *Aspergillus oryzae* gives "miso".

Rhizopus microsporus var. *chinensis*, *Aspergillus oryzae*, *Trichoderma pseudokoningii* release various exogenous enzymes able to hydrolyse cell wall of vegetables and seeds. Consequently, these strains releasing in particular cellulases, hemicellulases, and xylanases are used to degrade various fibers and improve digestibility of vegetable proteins. The use of these strains to improve the digestibility of seaweeds and in particular *Palmaria palmata* had to be evaluated.

The nitrogen release obtained by pepsin pancreatin digestion according to Savoie & Gauthier (1986) shows a good correlation with the *in vivo* digestibility and a good prediction of the nutritional value (Rozan *et al.*, 1997).

The objective of this paper is to examine the improvement of the *in vitro* digestibility of algal proteins after fermentation of *Palmaria palmata* by *Rhizopus microsporus* var. *chinensis*, *Aspergillus oryzae*, and *Trichoderma pseudokoningii*. Xylose content after 6-hour digestion of the samples and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of algal soluble protein extract after 6-hour digestion are used to characterize the effects of the fermentations.

Material and methods

Palmaria palmata was collected in autumn 1999 in Belle-Île, located on the French Brittany coast. Epyphytes were removed and samples were successively rinsed with seawater and distilled water. *Palmaria palmata* was freeze-dried for storage before use.

Three microscopic moulds were used: *Rhizopus microscopus* var. *chinensis* was identified by our group (Schwertz *et al.*, 1997) and is available under IHEM No. 6048 from the culture collection of the Institute of Hygiene and Epidemiology Mycology (rue Juliette Wystman 14, B-1050 Bruxelles, Belgium). *Aspergillus oryzae* NRRL 1988 was purchased (Northern Regional Research Laboratories, Peoria, Ill.). *Trichoderma pseudokoningii* was a wild strain, gracefully given by Pr Lin (Taiwan).

Nitrogen was determined by micro Kjeldahl procedure according to the AOAC method (AOAC, 1984).

Fermentations

Freeze-dried crude *Palmaria palmata* (A) were washed in demineralized water to remove salts. After washing, algae were freeze-dried (B1-8, Christ Bioblock, Hercules, CA, USA) (WA).

Then, WA was air-dried at 110°C during 30 min mixed with phosphate-citrate buffer, incubated 6 days at 37°C, finally heated 15 min at 100°C and grinding with ultraturax (Polytron, Switzerland) giving UHWA which is the fermentation blank.
A buffer pH 4.7, Na_2HPO_4, $12H_2O$ 0.2 M and citric acid 0.1 M, in distilled water (4/10, v/v) was used.
Three grams of UHWA were added in a Petri box, previously sterilized with buffer. One box received no strain (fermentation blank). The remaining boxes were sowed with 107 spores of *Rhizopus microsporus* var. *chinensis*, or *Aspergillus oryzae*, or *Trichoderma pseudokoningii*. The four Petri boxes were incubated at 37°C during 6 days excepted for the box containing *Trichoderma pseudokoningii* incubated during 14 days.
The fermentations were stopped by heating at 100°C during 15 minutes. Then, nitrogen estimation was realized according to the micro-Kjeldhal method and the samples were submitted to *in vitro* digestion.

In vitro digestion

In vitro digestion measurements were carried out on five samples (three fermented samples, fermentation blank and crude algae).
The multi-enzymatic method described by Savoie & Gauthier (1986) was used.
Dialysates were collected continuously with a peristaltic pump, each hour during 6 hours and are assayed for nitrogen.
Digestibility was calculated and was represented by nitrogen release. The relative digestibility is the expression of sample digestibility in fonction of casein digestibility.
Three *in vitro* digestion assays were carried out for each samples.

Determination of the xylose content

The extraction and the hydrolysis of hemicelluloses with dilute acid were carried out according to Southgate. Pentoses were assayed by a colorimetric method according to Southgate (Southgate DAT, 1991).

Polyacrylamide gel electrophoresis

The protein extracts of *Palmaria palmata* fermented by each of the three strains of moulds and crude alga (A) were submitted to SDS-PAGE to study the influence of the nature of the fermentation process on the protein digestibility. These extracts were obtained by double extraction by osmotic shock. Proteins were isolated by using 80% ammonium sulphate. SDS-PAGE was performed with a Mini-protean III electrophoresis unit (Bio-Rad, Hercules, CA, USA). The electrophoresis was performed with a gradient gel 4% - 15% in Tris-HCl 25 mM, pH 8.3, glycine 192 mM, SDS 0.1%. The separation was carried out at 200 V during 30 minutes. After separation, protein bands of algal extracts were detected by silver staining (Silver Stain Plus Kit, Biorad, Hercules, CA, USA).

Results

The effects of fermentation processes on *in vitro* digestibility of *Palmaria palmata* are illustrated in the table. The digestibilities of the fermented samples were significantly different among them, *Trichoderma pseudokoningii* being the most efficient to improve the protein digestibility. The soluble xylan content measured as xylose in the digestion cell after *in vitro* digestion is reported in figure 1. Fermentation with *Rhizopus microsporus* var. *chinensis* produced a similar level of residual xylose that fermentation blank (60.2 ± 5.3 vs 52.9 ± 3.0) while *Trichoderma pseudokoningii* was the best efficient mould to decrease xylose (18.8 ± 0.5). The electrophoretic patterns of the extracted proteins obtained from *Palmaria palmata* submitted to three different fermentations or washed, then submitted to 6-hours digestion were compared to that of the extracted proteins obtained from ground crude *Palmaria palmata* (fig. 2). A disappearance of high molecular size proteins was observed in both fermented an washed samples. The molecular size for the remaining bands were 30.5 kD for fermented samples.

Relative digestibilities and xylose content of different samples.

	Relative digestibility	Xylose content	SD relative digestibility	SD xylose content
Casein	100		0.500	
Tchrichoderma (Tch)	65.56	18.800	1.000	0.5
Asperpillus (Asp)	50.61	33.600	6.500	3.1
Rhizopus (Rhz)	45.00	60.100	7.400	5.3
Ferm. blank	36.28	52.900	1.500	3
Crude alga	19.65		1.900	

SD: Standard Deviation.

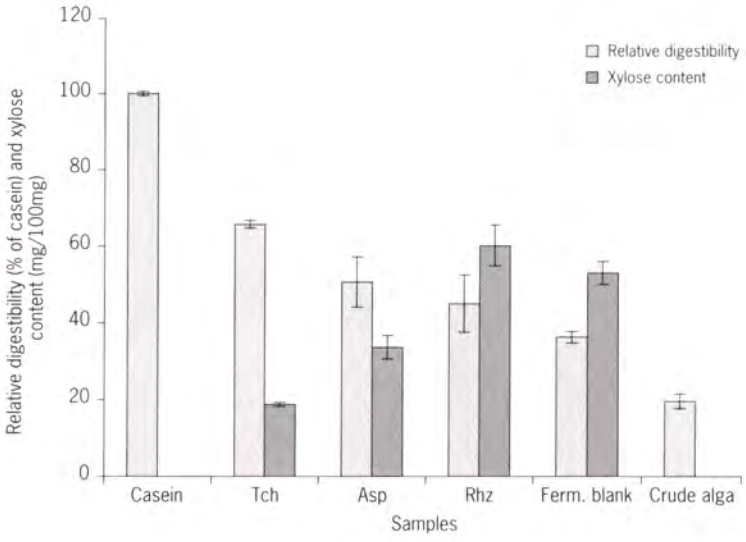

Figure 1
Relative digestibilities and xylose content of different samples.

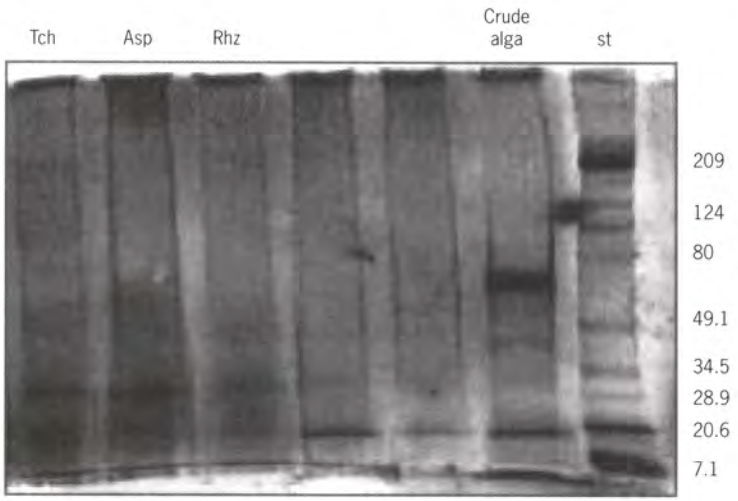

Figure 2
Electrophoretic profiles of fermentation samples and crude *Palmaria palmata*.

Discussion

A previous work showed the weak *in vitro* digestibility of crude *Palmaria palmata* (Galland-Irmouli *et al.*, 1999). Our results confirm that crude *Palmaria palmata* is very weak compared to casein (19.5% of relative digestibility). *Palmaria palmata* is a source of dietary vegetable protein with a great potential and its nutritional quality has to be improved. The fibers, representing at least 30% of the dry weight, and in particular the water-soluble xylan, are able to prevent the proteolysis and could explain the weak digestibility (Bobin-Dubigeon *et al.*, 1997). Consequently, to increase the nutrional value of *Palmaria palmata* protein, it is necessary to eliminate the fibers as much as possible.

The effect of fermentation by moulds process on protein digestibility was presently evaluated.

Compared to crude *Palmaria palmata*, the products of fermentation by *Rhizopus microsporus* var. *chinensis*, *Aspergillus oryzae* and *Trichoderma pseudokoningii* showed an unequally improved digestibility. The improvement varied in function of the strains used. *Trichoderma pseudokoningii* led to the best digestibility improvement and *Rhizopus microsporus* var. *chinensis* to the worst. After 6 hours, the digestibility of *Palmaria palmata* fermented by *Trichoderma pseudokoningii* reached 65.5% of that of casein.

Aspergillus oryzae is used in Japan, Taiwan and Malaysia to manufacture miso or soya sauce. It produces different enzymes such as glycosidases, peptidases, proteases. Among theses glycosidases, some authors have described endo- and exo-xylanase, ß-glucanase and ß-galactosidase (Bakalova *et al.*, 1996). *Trichoderma* strains produce also glycosidases such as endoxylanase, ß-glucanase and ß-glucosidase, cellobiohydrolase (Huang *et al.*, 1991). *Rhizopus microsporus* var. *chinensis* is used in Indone-

sia for the preparation of tempeh, a traditional soya bean product. *Rhizopus microsporus* var. *chinensis* produces different enzymes such as proteases and glycohydrolases.

The release of different enzymes by the different moulds can explain the different improvements of digestibility of alga, because these enzymes can degrade the polysaccharides remaining after the washing which eliminates the soluble fibers. The efficacity of these enzymes to degrade polysaccharides was evaluated by the xylose content of the different samples. The improvement of the digestibility of *Palmaria palmata* seems to be related to the efficiency of the strains to hydrolyse polysaccharides for their growth.

The SDS-PAGE protein patterns of the three extracts of fermented samples after-6 hour digestion are very similar although their digestibilities are significantly different. This suggests that proteolytic enzymes are equally effective on the soluble proteins of dulse but not on the insoluble proteins whose the hydrolysis is related to their interactions with the remaining fibers after fermentations.

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Bioactive bacterial exopolysaccharides: modification, characterization and preliminary results on biological activity

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Abstract

In recent years, there has been a growing interest in the isolation and identification of new microbial polysaccharides that might have new uses in many industrial sectors. Bacterial polysaccharides offer potential applications for the pharmaceutical industry. They compete with polysaccharides from other sources such as seaweeds (alginates, carragheenans), crustaceans (chitin), animals (glycosaminoglycans) or plants. Bacteria associated with deep-sea hydrothermal conditions have demonstrated their ability to produce in an aerobic carbohydrate-based medium, unusual extracellular polymers. With the aim of promoting biological activities, chemical modifications (depolymerization and substitution reactions) of three described exopolysaccharides (EPS) have been undertaken. Characterization and preliminary results on anticoagulant activities of the modified EPS from deep-sea hydrothermal vents showed that only sulphated derivatives were active, but not native EPS. Moreover, the three sulphated derivatives presenting differences in structural feature were endowed with original anticoagulant properties compared to heparin. They presented a lower anticoagulant activity than heparin so could be promising new antithrombotic drugs without major bleeding risk.

Introduction

Marine microorganisms offer a rich source of polysaccharides with novel structures. Since 1994, new bacteria have been searched for near deep-sea hydrothermal vents characterized by extreme pressure and temperature conditions (Guézennec *et al.*, 1994; Rougeaux *et al.*, 1996). Interest in mass-culture of microorganisms from the marine environment has increased considerably, representing an innovative approach to the biotechnological use of under-exploited resources (Sudo *et al.*,

1995). Some thermophilic and mesophilic EPS-producing strains have been isolated from deep-sea hydrothermal vents (Vincent *et al.*, 1994; Guézennec *et al.*, 1994). Some bacteria (e.g. *Pseudoalteromonas* or HYD 721 strain and *Vibrio diabolicus* or HE 800 strain) found in these conditions have produced extracellular polymers with original structures when grown in an aerobic carbohydrate-based medium. The HYD 721 EPS consists of a branched octosaccharide repeating unit rich in neutral sugars (Rougeaux *et al.*, 1999a) and the repeating unit of the HE 800 EPS is a linear tetrasaccharide with the following composition: →3)-β-D-GlcpNAc-(1→4)- β-D-GlcpA-(1→4)- β-D-GlcpA-(1→4)-α-D-GalpNAc-(1→ (Rougeaux *et al.*, 1999b). *Alteromonas infernus* or GY 785 strain, a new species of bacterium isolated quite recently, secretes a water-soluble acidic heteropolysaccharide consisting of glucose, galactose, glucuronic and galacturonic acids. The composition of this high-molecular-weight polysaccharide differs in monosaccharide content and/or ratio and sulphate content (10%) from the other EPS isolated from deep-sea hydrothermal bacteria (Raguénès *et al.*, 1997). But all described EPS are high-molecular-weight polymers (106 g/mol) and usually slightly substituted. In our previous studies, highly sulphated low-molecular-weight (LMW) EPS derivatives were obtained from the GY 785 EPS after combined sulphation and depolymerization (acid hydrolysis or free radical depolymerization), without altering the osidic composition of this acidic polymer (Guézennec *et al.*, 1998; Colliec-Jouault *et al.*, 2001). In the present study, the preparation of new heparinoids or heparin-like compounds from the three high-molecular-weight EPS described above (HYD 721, HE 800 and GY 785) have been undertaken. The characterization, the anticoagulant activity and the interaction with serpins of the sulphated LMW EPS derivatives have been performed.

Material and methods

Isolation and preparation of the EPS derivatives

The three EPS (HE 800, HYD 721 and GY 785) were produced, purified and characterized as previously described (Guézennec *et al.*, 1994, 1998; Vincent *et al.*, 1994; Raguénès *et al.*, 1997). Briefly, strains were placed in 2-litre fermenter and exopolymer production was performed at room temperature under atmospheric pressure in marine 2216 broth supplemented with glucose, peptone and yeast at pH 7.2. After 2 days, the viscosity of medium remains stable, the concentration of exopolymer in medium is maximal. The EPS was isolated from culture medium by high-speed centrifugation to remove bacterial cells and precipitated from the supernatant with ethanol.

The high-molecular-weight EPS were depolymerized by a free-radical reaction according to a previously described procedure (Colliec-Jouault *et al.*, 2001). Depolymerized EPS were chemically sulphated according to a previously described sulphation procedure (Guézennec *et al.*, 1998),

the sulphation of either sodium or pyridinium salt of EPS was performed with pyridine-sulphur trioxide in DMF. The depolymerized HE 800 only was treated with sodium hydroxide solution to obtain N-deacetylated HE 800 prior sulphation.

Characterization of the EPS derivatives

The EPS derivatives molecular mass determination (number-average, Mn and weight-average, Mw) and polydispersity, I = Mw/Mn was performed by high-performance steric exclusion chromatography (HPSEC) using a Superdex column (Pharmacia PC 3.2/30) and Aramis software (JMBS Développements, Le Fontanil, France) as previously described (Chevolot *et al.*, 1999; Colliec-Jouault *et al.*, 2001). Elemental analysis (C, H, N and S) was performed by "Le Centre de Microanalyse" of the CNRS (Gif-sur-Yvette, France). Monosaccharide content was determined by analysis of trimethylsilyl derivatives after methanolysis by gas chromatography (GC) as previously described (Montreuil *et al.*, 1986; Rougeaux *et al.*, 1999b).

Clotting assays

Activated partial thromboplastin time (APTT) with the APTT Organon kit (Organon Technika, France) and thrombin clotting time (TT) with purified human thrombin 5 NIH U/ml were performed as previously described (Mauray *et al.*, 1995).

Electrophoretic analysis of serpin binding

The binding of polysaccharides to human antithrombin (AT) and human heparin cofactor II (HCII) was analyzed by affinity co-electrophoresis according to a previously described technique (Lee & Lander, 1991) and detailed procedure is reported elsewhere (Colliec-Jouault *et al.*, 2001). Briefly, the polysaccharides were electrophoresed in agarose gels through zones containing AT or HCII. At neutral pH, the electrophoretic mobilities of polysaccharides are much higher than those of serpins, and the binding of polysaccharides to serpins decreases their electrophoretic mobility.

Results and discussion

Characterization of the EPS derivatives

The molecular mass and chemical composition of each EPS derivative were summarized in table 1. Homogeneous low-molecular-weight polysaccharides were obtained without any change in their respective initial osidic composition. The EPS derivatives had a sulphur content above 10% corresponding to 30% sulphate groups as found in glycosaminoglycan family.

Table 1 - Molecular mass and chemical composition of the sulphated LMW EPS derivatives.

	Mw[a] (g/mol)	I[a] (Mw/Mn)	S[b] (%)	Neutral sugars[c] (%)	Uronic acids[c] (%)	Hexosamine[c] (%)
HE 800 derivative	6,000	1.2	12.5	0.4 ± 0.1	15 ± 1	18 ± 2
HYD 721 derivative	20,000	2	10	53 ± 4	6 ± 1	0
GY 785 derivative	24,000	1.3	12	24 ± 1	10 ± 2	0

a) Mw (weight-average molecular mass), Mn (number-average molecular mass), and I (polydispersity) were measured by HPSEC using pullulans as standards. b) Elemental analysis. c) GC analysis of trimethylsilyl derivatives after acidic methanolysis.

Anticoagulant properties of the EPS derivatives in clotting assays

The *in vitro* anticoagulant activity of the EPS derivatives was first measured in APTT and compared with this of a low-molecular-weight (LMW) heparin (fig. 1). This assay is a global clotting time that explores the intrinsic pathway of blood coagulation which leads to thrombin formation. The native exopolysaccharides did not prolong the clotting time; indeed the clotting values obtained in their presence were similar to control values (40 seconds). The three sulphated derivatives were able to prolong the APTT but they were less anticoagulant than the LMW heparin. The concentrations required to double the control time were between 10 and 15 µg for the derivatives compared to 4 µg for LMW heparin. GY 785 derivative was more anticoagulant than the other derivatives. The anticoagulant activity of the EPS derivatives was then

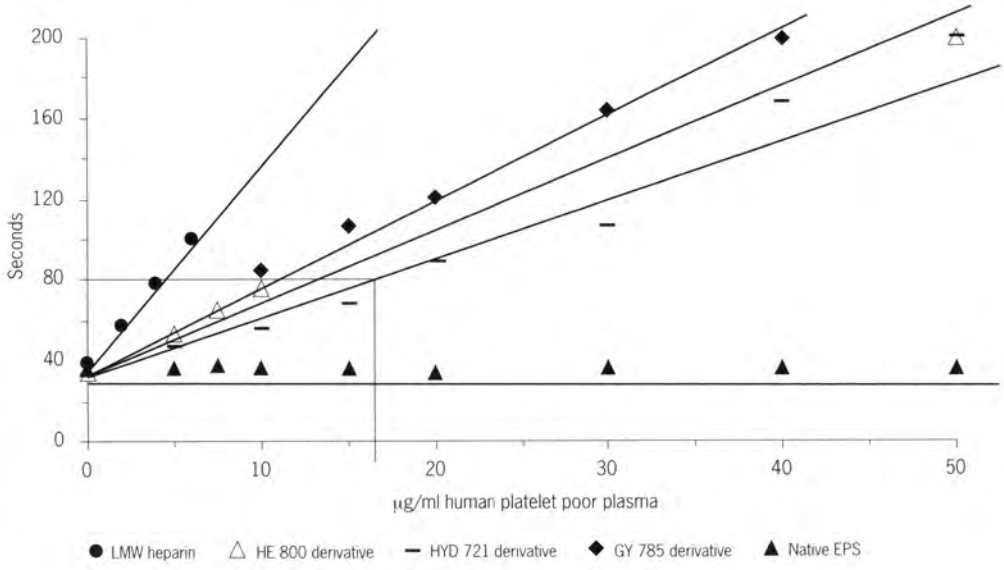

Figure 1
Anticoagulant activity measured by activated partial thromboplastin time (APTT). Polysaccharides tested after dilution in human platelet poor plasma at various concentrations, assay performed with Organon APTT kit; and results are means of 4 experiments.

measured by using an other clotting assay, thrombin time, and compared with this of a LMW heparin (tab. 2). The inhibition of thrombin, a key factor of blood coagulation, in the presence of human plasma is observed in this assay. Compared to LMW heparin, the EPS derivatives had a weak effect on thrombin time. As observed in APTT, GY 785 is more anticoagulant than the other derivatives.

Table 2 - Anticoagulant activity: thrombin time.

	Thrombin time[a] (seconds)			
$\mu g/mL$ PPP[b]	0	10	50	100
HE 800 derivative	15 ± 0.6	22.6 ± 0.1	108.7 ± 2	> 200
HYD 721 derivative	15 ± 0.6	21 ± 1	166.4 ± 5	> 200
GY 785 derivative	15 ± 0.6	25.5 ± 0.5	> 200	> 200
$\mu g/mL$ PPP[b]	0	2	4	6
LMW heparin	15 ± 0.6	96 ± 8	> 200	> 200

a) Purified human thrombin (5 NIH U/ml). Results are expressed as means + SD (n=4).
b) PPP: Platelet poor plasma.

Analysis of the interaction of the EPS derivatives with serpins

Coagulation factors are serine proteinases and serpins (serine proteinase inhibitors) present in human plasma play a major role in the regulation of coagulation, fibrinolysis and inflammation. Several plasma serpins bind to sulphated polysaccharides such as heparin and dermatan sulphate. Antithrombin and heparin cofactor II, thrombin inhibitors, undergo activation in the presence of heparin. The interaction of the three sulphated derivatives with serpins was studied by affinity co-electrophoresis (fig. 2). This technique allows both polysaccharides and proteins to migrate freely during electrophoresis. In the conditions used, the mobility of the polysaccharide chains was higher than that of protein. So without protein (tracks 0) all chains migrated to the bottom of the gel giving a single spot. In the presence of AT (fig. 2A and tracks AT), as described in literature, 30% of heparin chains were delayed and strongly bound to AT; consequently the migrating heparin front was split by AT into two distinct spots. Only the GY 785 derivative bound to AT and all chains were delayed giving a single spot. In the presence of HC II (fig. 2B, tracks HC) only few chains of the GY 785 derivative strongly interacted with the protein. With the LMW heparin and other EPS derivatives, the migration front was similar to the control (buffer).

In summary, these preliminary results show that the three sulphated derivatives, presenting differences in structural feature, have an inhibitory effect on the intrinsic pathway of the coagulation. But only the GY 785 derivative presents a high affinity to AT, and consequently as heparin, could catalyze the inhibition by AT of several coagulation factors. It will be interesting now to perform reptilase time in the presence of the three derivatives to determine if they have an effect on the fibrin network formation.

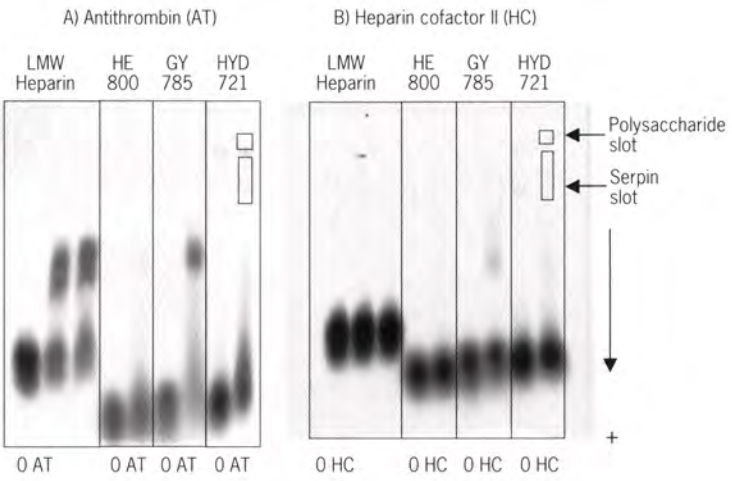

Figure 2
Analysis of EPS derivative-antithrombin binding (A) and EPS derivative-heparin cofactor II binding (B). Electrophoresis of LMW heparin or EPS derivatives (20 µg) through zones containing buffer (0) or serpin (86 µM AT or 1.5 µM HC).

Conclusion

The chemical modifications (radical depolymerization and sulphation) of the exopolysaccharides provide a new source of heparinoids or heparin-like compounds presenting different structural features. The EPS derivatives are less efficient than LMW heparin in clotting assays; they present differences in affinity and structural selectivity in the binding of serpins. This newly discovered EPS of bacterial origin have original sulphation patterns after modification so they can be used to constitute a library of compounds usefull for elucidation of the structure-activity relationship. As fucan, a matrix sulphated polysaccharide extracted from brown seaweeds, the EPS derivatives with a lower anticoagulant activity than heparin could exhibit a potent antithrombotic effect with a low bleeding risk.

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Chapter IV

Enzymes, proteins and peptides

Post mortem proteolysis in sea bass muscle

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Introduction

Muscle post mortem evolution is characterized by successive biochemical reactions resulting in the disorganisation of the myofibrillar structure. These autolytic changes may affect various aspects of raw fish quality. There is good evidence in the literature that most of these changes are due to proteolysis but an understanding of the involved mechanisms has not reached general agreement. The aim of our research was to investigate the mechanisms of the post mortem degradation in fish flesh. The breakdown of fish muscle proteins is probably caused by several categories of proteases acting synergistically (mainly lysosomal acidic cathepsins and neutral calcium-dependent calpains). Up to now, our research focus has been directed towards calpains.

Observed changes in post mortem fish muscle proteins

These changes can vary depending on the range of factors such as species, physiological status, stress prior to death, sex, age, reproduction period and temperature of post mortem storage. The weakening and the disorganisation of the Z line structure, the detachment of sarcolemma as well as the degradation of titin, nebulin and dystrophin have already been observed in post mortem sea bass muscle (Astier *et al.*, 1991; Papa *et al.*, 1997).

Our studies on sea bass post mortem protein changes have shown, in particular, the release and partial proteolysis of α-actinin (Papa *et al.*, 1996), which cross-links elastic filaments to thin filaments of actin. No change in desmin, a myofibrillar packaging protein, was noted in this fish muscle (Verrez-Bagnis *et al.*, 1999). SDS-PAGE on sea bass sarcoplasmic proteins revealed the gradual disappearance of a protein band of 16 kDa immediately after fish death (Verrez-Bagnis *et al.*, 2001). This protein has been identified as a nucleoside diphosphate kinase after amino acid sequencing.

Proteases and proteolytic action on muscle proteins

Calpains, calcium-dependent neutral proteases, are usually described as the proteases capable of carrying out the initial step in myofibrillar proteolysis. Three calpain isoforms have been found in sea bass; the first one is a μ-calpain (A) and the two others are m-calpains (B, C).

Their respective amounts reach their maximum during the spawning period and one isoform is completely lacking in winter (fig. 1). This could be related to the variation, from season to season, of the extent or the rate of muscle degradation.

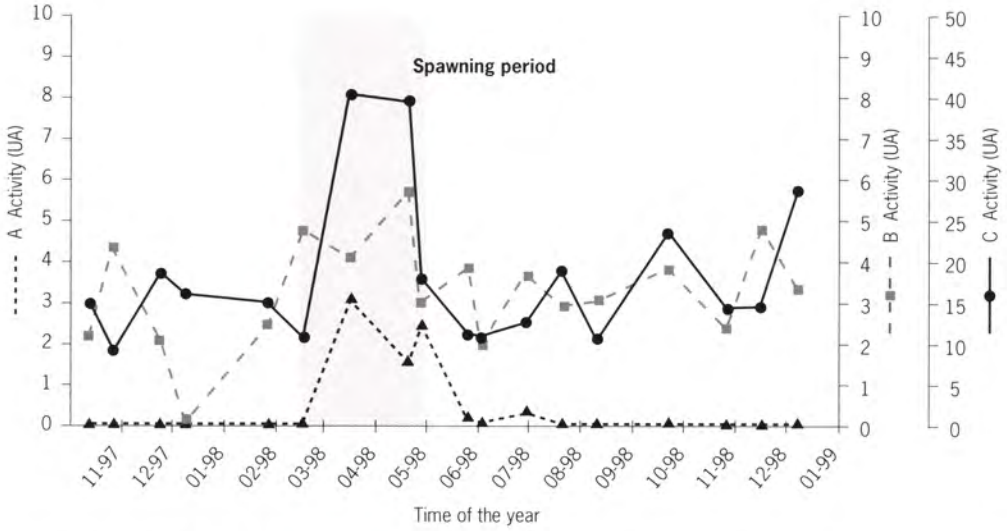

Figure 1 - Seasonal activity level of sea bass calpains. For each living animal (one or two per month), calpains were extracted from the white muscle and partially purified by DEAE-Sepharose chromatography. They were resolved into three peaks.

In vitro, the major m-calpain (peak C) was able to release myofibrillar components such as α-actinin and tropomyosin in soluble fraction and to degrade myosin heavy chain, α-actinin and desmin (fig. 2). A 26.5 kDa sarcoplasmic component was also degraded by calpain (fig. 3).

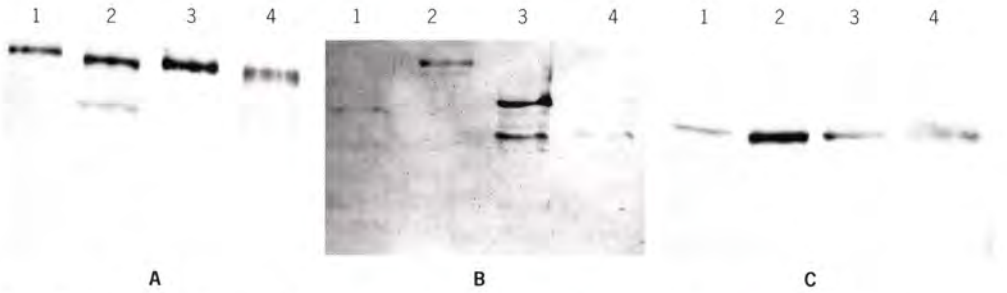

Figure 2 - Immunoblots showing the degradation or release of α-actinin (A), desmin (B), tropomyosin (C) in myofibrils by m-calpain. Lane 1: supernatant at 0 min, lane 2: supernatant at 120 min, lane 3: pellet at 0 min, lane 4: pellet at 120 minutes.

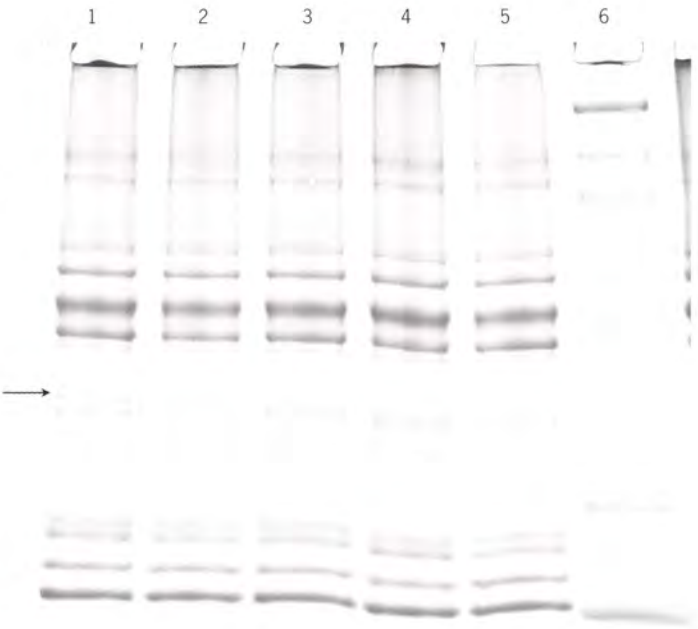

Figure 3
Degradation of a 26.5 kDa sarcoplasmic component by m-calpain (120 min incubation) monitored by SDS-PAGE. Lane 1: initial sample, lane 2: 15 min of incubation, lane 3: 30 min of incubation, lane 4: 60 min of incubation, lane 5: 120 min of incubation, lane 6: molecular weight standards (from top of the gel: 203, 118, 82, 50.4, 33.4, 26.7, 19.6, and 7.4 kDa).

Conclusion

Knowledge of the proteolytic mechanisms underlying post mortem evolution is fragmented and often contradictory. We have shown here the ability of m-calpain to degrade sea bass muscle proteins. Based on our observations (tab.), it is however unlikely that calpain acts on myofibrils in post mortem sea bass muscle since *in vitro*, it is able to degrade desmin, myosin heavy chain and the 32 kDa component which remain stable in the muscle.

Comparison between the evolution of sea bass muscle proteins during post mortem storage and the *in vitro* effect of m-calpain on sea bass muscle proteins.

	Post mortem	m-calpain
MHC	ST	D
α-actinin	R-D	R-D
Desmin	ST	D
Actin	ST	ST
Tropomyosin	ST	R
32 kDa	ST	D
< 22 kDa		R

D: Degraded; R: Released in the soluble fraction; ST: not degraded.

To improve our analysis, we should take into account the evolution of the other huge proteins such as dystrophin, titin and nebulin (Astier *et al.*, 1991; Papa *et al.*, 1997). Furthermore, if calpains are known to be active just after death, other proteases like cathepsins are also reported to be implicated in post mortem changes; in particular, calpains have been believed to initiate the proteolytic degradation of myofibrils making proteins more susceptible to cathepsins (Goll *et al.*, 1992). Their role in sea bass proteins degradation should be evaluated.

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An example of shrimp waste upgrading: the production of hydrolysates with antioxidative-free radical scavenging properties

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Abstract

The demand for new materials and products from marine origin is still growing. Hydrolysates from seawater fish species and marine invertebrates have already revealed interesting characteristics such as bioactive peptides exhibiting hormonal, growth stimulatory and immuno-modulatory functions, peptides with opioid-like activity or peptides inhibiting the angiotensin I-converting enzyme(*). In this study, we have explored the ability of shrimp waste hydrolysates to exhibit antioxidative properties. Shrimps wastes were solubilized and separated through a series of filtrations and chromatographic steps. The antioxidative activity was investigated using the beta-carotene/linoleate model and the DPPH test. From size exclusion chromatography studies, it was estimated that a peptidic fraction of molecular weight ranging from 400 to 300 Da contained the strongest antioxidative activity. Shrimp waste hydrolysates could have promising practical applications in various areas, including the nutraceutical field, which presents rising interest.

Introduction

The shellfish industry generates a significant amount of solid wastes: indeed, it has been estimated that, in commercial processing, the meat recovery constitute about 25% (w/w). The solid wastes containing 25-30% dry matter are tissue proteins, minerals and chitin. These wastes have an appreciate potential for pollution and do pose a disposal problem (Gildberg & Stenberg, 2001; Ferrer *et al.*, 1996). The recent advances in marine biotechnology have demonstrated the capacity of fish and crustacean by-products to represent potential resources for bioactive materials. Actually, the proteic part of shellfish residues has not been fully exploited and could be recovered and up-graded as a rich source of amino acids, peptides, proteins and other useful chemicals.

(*) Refers to the available results recently obtained within the framework of the European project FAIR Hydrofish CT 97-3097.

The feasibility of upgrading waste materials from capture fisheries by using commercial proteolytic enzymes generating a range of hydrolysates for use a variety of applications was demonstrated within the European research programme FAIR Hydrofish CT 97-3097. More specifically, it was shown that it is possible to control the hydrolytic process to tailor the product in respect of the hydrolysis degree achieved and, therefore, the size profile of molecules in the hydrolysates. The enzymatic hydrolysates from fishery wastes and by-products have proved to contain biologically active factors such as immunostimulating substances (Gildberg *et al.*, 1998) and small gastrointestinal peptides like gastrin and cholecystokinin (Ravallec-Plé *et al.*, 2000). Several other classes of molecules have been generated such as CGRP activity, opioid-like activity or peptides inhibiting the ACE activity (Rousseau *et al.*, 2001). Fish protein hydrolysates have also been shown to be very good sources of microbial peptones (Dufossé *et al.*, 2001; Guérard *et al.*, 2001). However, within these bioactive activities, very little has been done about the antioxidative properties of extracts or hydrolysates from marine organisms.

According to a very general definition, antioxidatives are "substances capable of delaying, retarding or preventing oxidation processes" (Schuler, 1990). Hydrolysed proteins from soya beans, yeasts, casein and other sources have been tested in foods and model systems and found to posses marked antioxidative activity (Pratt & Hudson, 1990; Park *et al.*, 2001; Suetsuna *et al.*, 2000). Antioxidative compounds were also isolated from a pepsin digest of prawn muscle (Suetsuna, 2000), capelin hydrolysate (Amarowicz & Shahidi, 1997) and gelatine hydrolysate of Alaska pollack skin (Kim *et al.*, 2001). In addition, antioxidative compounds were identified in marine extracts prepared without enzymatic hydrolysis. Yoshikawa *et al.* (1997) prepared an oyster extract that directly scavenged superoxide and hydroxyl radicals. Several amino acids, such as Tyr, Met, His, Lys and Trp are generally accepted as antioxidatives despite their pro-oxidative effects in some cases. Many antioxidative peptides included hydrophobic amino acid residues, Val or Leu, at the N-terminus of the peptides (Kim *et al.*, 2001).

The objectives of this study were to hydrolyze shrimp wastes for the production of antioxidative compounds and to isolate and partially characterise these compounds.

Hydrolysis of shrimp wastes for the production of antioxidative substances

The hydrolysis experiments were carried out using the pH-stat method in a 500-ml glass reactor in controlled conditions (pH, temperature and stirring speed). Shrimp wastes were solubilised at pH 9.7 and 66°C using Alcalase® 2,4 l (Novo Industry) or without enzyme addition. When no enzyme was added, the nitrogen recovery (NR) values ranged from 46.8% to 62.4% after 15-min and 2-hour hydrolysis respectively.

When Alcalase® 2,4 l was added, the NR values ranged from 72.2 to 86.8% (tab. 1). These results indicated that the enzyme addition had a strong influence on the NR from the substrate studied. However, a chemical solubilisation of the substrate was also obtained without enzyme addition. This may be related to the drastic experimental conditions. The antioxidative efficacy of the hydrolysates was tested at the same protein concentration (5 mg/ml). The best protection (63,1%) against the beta-carotene bleaching was obtained from the Alcalase digest after 2-hour hydrolysis (tab. 2). The strongest activity using the DPPH test (87.25%) was obtained after 15 minutes of substrate solubilisation without enzyme (B15 assay). On an antioxidative efficacy basis using the DPPH test, the B15 sample was chosen for the further studies.

Table 1 - Nitrogen recovery (NR, %), protein concentration and total phenolic contents of hydrolysates prepared at pH 9.76 and 66.2°C.

	NR (%)*	[protein] mg/ml**	Phenolic compounds (mg/ml GAE***)
No enzyme added			
B0		5.3	318.2
B15 (15-min hydrolysis)	**46.8**	**6.2**	**411.8**
B 1 (1-hour hydrolysis)	58.8	7.9	502.7
B 2 (2-hour hydrolysis)	62.4	8.4	527.1
Alcalase (E/S=68.1 AU/kg proteins)			
O15 (15-min hydrolysis)	72.2	8.4	570.1
O 1 (1-hour hydrolysis)	83.8	8.9	644.0
O 2 (2-hour hydrolysis)	86.8	9.7	700.5

* NR (%) : percentage ratio of nitrogen content in the hydrolysate to that in the original substrate, the nitrogen was determined by the Kjeldahl method (AOAC, 1990);
** according to Lowry assay (Lowry *et al.*, 1951);
***GAE: Gallic acid equivalents (according to the Singleton & Rossi (1965) procedure).

Table 2 - Antioxidative activities of shrimp wastes solubilised at pH 9.76 and 66.2°C. The hydrolysates were tested at the same protein concentration (5 mg/ml).

	Antioxidative activity (%) (DPPH test)*	Antioxidative activity (%) (beta-carotene test)**
No enzyme added		
B0	55.3	31.7
B15 (15-min hydrolysis)	**87.3**	37.4
B 1 (1-hour hydrolysis)	77.3	54.3
B 2 (2-hour hydrolysis)	73.2	47.4
Alcalase (E/S=68.1 AU/kg proteins)		
O15 (15-min hydrolysis)	71.7	45.1
O 1 (1-hour hydrolysis)	74.7	56.4
O 2 (2-hour hydrolysis)	**74.0**	**63.1**
BHA (2,5 mg/ml)		94.8

* DPPH test (according to Morales & Jimenez-Perez, 2001);
** beta-carotene/linoleate model system (according to Marco, 1968).

Partial purification of the antioxidative fraction

The shrimp waste hydrolysate (B15 sample) was separated through a series of microfiltration (0.45 µm) and ultrafiltrations with MWCO 30 and 5 kDa. Three permeates were obtained and the antioxidative efficacy of the hydrolysates was investigated using the DPPH test. The figure 1 showed the molecular weight distribution of the 3 permeates from Superdex 75 column. The molecular weight distributions were in accordance with the MWCO size of the membranes used. From the DPPH test, the strongest antioxidative activity (74%) was measured in the fraction below 5 kDa.

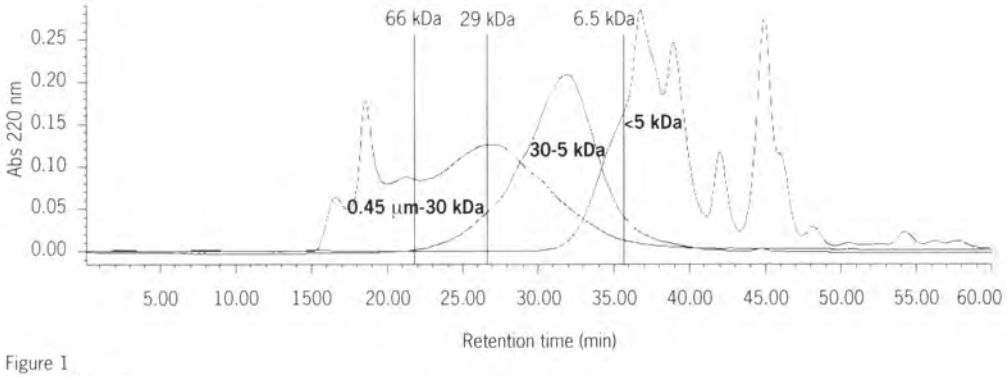

Figure 1
Molecular weight distributions of the 3 permeates from Superdex 75 column. Absorbance was monitored at 220 nm. The hydrolysates were successively filtered through a 0.45µm cut-off membrane and two ultra-filtration membranes which Molecular Weight Cut-Off (MWCO) were respectively 30 and 5 kDa.

Then the permeate from 5 kDa membrane was applied to a Sephadex G-25 column equilibrated with 50 mM phosphate buffer (pH 7.0). Six fractions (I-VI) were separated. The highest absorbance values at 220 nm was noted for fraction II which molecular size distribution was estimated to be ranged from 400 to 300 Da. Each fraction was collected. Application of fractions I to VI to DPPH and beta-carotene tests resulted in the detection of a strongest antioxidative activity in fraction II, although the protein concentration was very low (0.23 mg/ml) (tab. 3).

Table 3 - Protein concentration, total phenolic content and antioxidative activities of the fractions separated by Sephadex G-25 chromatography.

Fractions	Antioxidative activity (%) (DPPH test)*	Antioxidative activity (%) (beta-carotene test)**	Protein*** (mg/ml)	Phenolic compounds (mg/ml GAE****)
I	5.19	18.84	0.31	6.24
II	29.76	77.24	0.23	9.88
III	6.64	38.94	0.13	7.11
IV	5.21	11.48	0.22	37.58
V	4.05	10.71	0.01	0.79
VI	5.23	10.29	0.2	5.55

* DPPH test (according to Morales & Jimenez-Perez, 2001); ** beta-carotene/linoleate model system (according to Marco, 1968); *** according to Lowry assay (*Lowry et al.*, 1951); **** GAE: Gallic acid equivalents (according to the Singleton & Rossi (1965) procedure).

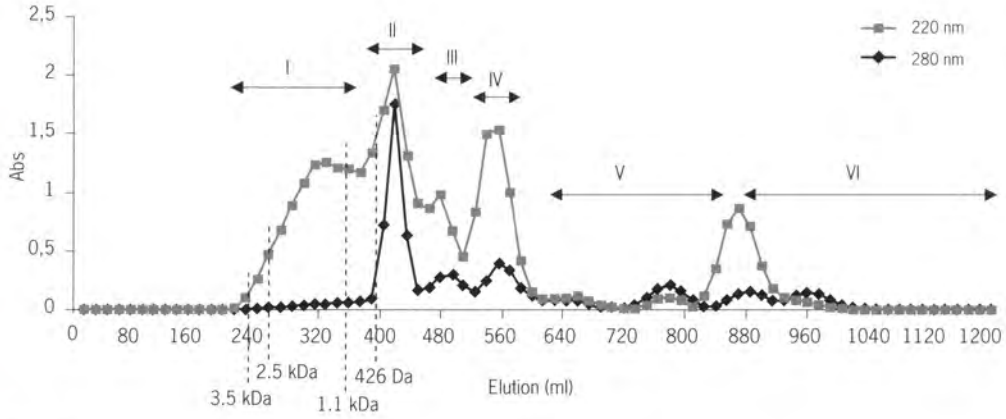

Figure 2
Separation of 5 kDa permeate by Sephadex G-25 column chromatography equilibrated with 50 mM phosphate buffer. Absorbance was monitored at 220 and 280 nm. Samples were diluted 5 times before absorbance readings.

Fraction II exhibiting the strongest efficacy was lyophilised and subjected to cation exchange chromatography on a SP Sephadex C-25 column equilibrated at pH 4. Three fractions (II-1, II-2 and II-3) were separated during the step of column washing with 20 mM sodium phosphate buffer pH 4.0 (fig. 3). This result suggested the anionic character of the three peptidic fractions. Fraction II-1 was found to possess the strongest antioxidative activity measured by DPPH test at a very low protein concentration (tab. 4). Fractions II-1 and II-2 also showed an efficacy against the oxydation of beta-carotene. Fractions II-1 and II-2 did not strongly absorbed at 280nm, which could indicate the absence of amino acid residues such as Tyr or Trp or other aromatic compounds in these peptides. Fraction II-3 did possess these compounds but its antioxidative activity was lower than those of the two other fractions.

Figure 3
Rechromatography of fraction II on cation exchange SP-Sephadex C-25 column previously equilbrated with 20 mM sodium phosphate buffer (pH 4.0). The column was washed with the same buffer.

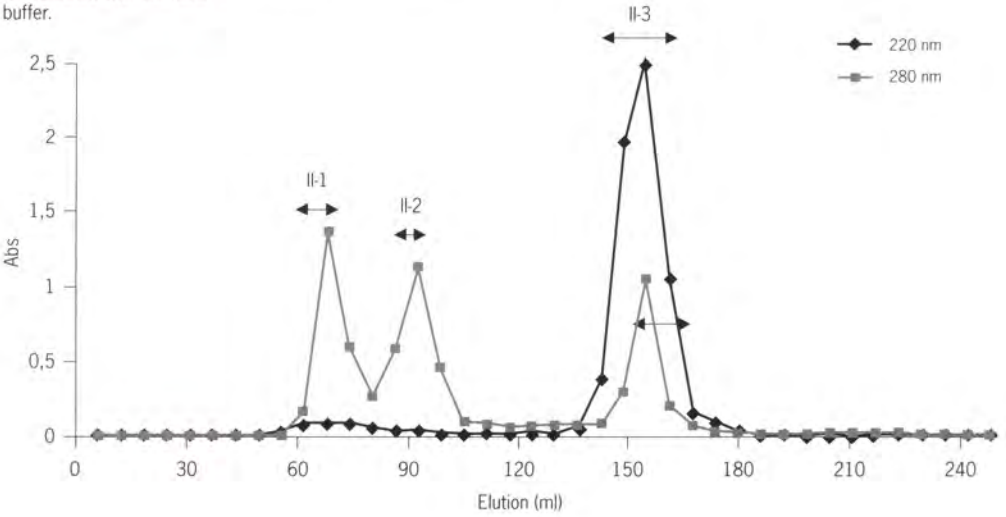

Table 4 - Protein concentration, total phenolic content and antioxidative activities of the fractions separated by SP Sephadex C-25 chromatography.

Fractions	Protein* (µg/ml)	Antioxidative activity (%) (beta-carotene test)**	Antioxidative activity (%) (DPPH test)***
II-1	2.3	40.83	43.07
II-2	2.1	48.74	11.04
II-3	3.6	13.31	8.34

* according to Lowry assay (Lowry *et al.*, 1951);
** beta-carotene/linoleate model system (according to Marco, 1968);
*** DPPH test (according to Morales & Jimenez-Perez, 2001).

Conclusion

We partly purified antioxidative compounds from shrimp wastes solubilized at pH 9.7 and 66°C. The molecular weight of the peptidic fraction was estimated to be ranged from 300 to 400 Da. Further studies will focus on the purification and the sequence identification of the small peptide(s) involved in the antioxidative activity.

Although phenolic compounds are well-known antioxidatives, the antioxidative activity may be attributed to amino acids and peptides and their association with other constituents in the hydrolysate. Thus, many protein hydrolysates have an antioxidative activity, but the active substances and the mechanism of action of many of them are still unknown. The structure-activity relationships of antioxidative peptides need to be well defined.

The economy of industrial processing of crustaceans could be improved by the full utilisation of wastes, which constitute good sources of both chitin and proteins. The increase of antioxidative compounds in hydrolysates could be obtained at a pilot scale by optimising the separations using membranes in aqueous and/or organic phases. These antioxidative compounds could have promising applications in various areas, including the nutraceutical and cosmetic fields.

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Crassostrea gigas peptic hydrolysate: a source of various biologically active molecules

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Abstract

Crassostrea gigas from Atlantic coast were digested by pepsin at pH 2 and 40°C during 3 hours. Using Guinea pig ileum test, presence of opioid molecules was assessed. The hydrolysate was only able to decrease partially Guinea pig ileum electrically induced contractions. Only a pseudo-opioid effect was observed. The anti-hypertensive potential of the hydrolysate was checked using the ability of the hydrolysate to inhibit the Angiotensin converting enzyme (ACE). This enzyme is partially responsible of hypertension. Only 20 minutes of hydrolysis allowed the production of an hydrolysate able to inhibit 90% of ACE activity. The concentration of hydrolysate needed to inhibit 50% of ACE activity (IC_{50}) was 72mg/l. Peptic oysters hydrolysate was analysed on RP-HPLC and 7 fractions were manually collected. ACE test was performed on each fraction. The fraction 6 was the most efficient since it had an IC_{50} of 5.3mg/l. Molecules contained in fraction 6 should be identified and tested *in vitro* and *in vivo*. No anti-oxidant activities were evidenced in oysters hydrolysate fractions obtained by RP-HPLC but two fractions exhibited interesting antiviral effects against herpes virus type 1. Further work will concern mainly anti-hypertensive potential of peptic oysters hydrolysate fractions and search of antiviral molecules. Such oysters hydrolysates produced by a classical gastric enzyme could be of interest for the emerging nutraceutical market.

Introduction

The purpose of this study was to check the presence of various bioactive molecules in oysters (*Crassostrea gigas*) harvested on the Atlantic coast. To release peptides from oysters proteins, grinded oysters were hydrolysed by pepsin according gastrointestinal conditions: pH 2, 40°C. Guinea pig ileum test, useful for search of relaxing (opioid) molecules, was performed on peptic oysters hydrolysate. We also looked for inhibitors of angiotensin I converting enzyme (ACE). The inhibition of ACE is important because this enzyme is involved in the Renin angiotensin system which regulates arterial blood pressure. Inhibition

of ACE reduces the production of angiotensin II (vasoconstrictor) and can increase bradykinin and enkephalin levels (vasodilators). Thus, inhibitors of ACE are potentially of great importance for controlling blood pressure and are currently used as anti-hypertensive agents.
ACE inhibitory activity of peptic oysters hydrolysate was followed along the hydrolysis process. The spectrophometrical Holmquist method (Holmquist *et al.*, 1979) was used for quantification of ACE inhibitory activity. The ability of peptic oysters hydrolysate to prevent β-carotene-linoleate bleaching induced by free radicals was also examined. This last test is useful for search of anti-oxidant activities. Finally, thanks to a collaboration, we also checked the presence of inhibitors of *Herpes simplex* virus in our oysters peptic hydrolysate.

Results and discussion

Oysters (*Crassostrea gigas*) were collected in Bouin (France), opened and grinded before being freeze dried. They were received as a gift from Philippe Roy and Annie Landrein, Ifremer Nantes.
Solution of 80 g oyster powder in 520 ml of distilled water was placed in a double coated reactor and heated at 40°C. The thick resulting solution was stirred by a propeller mixer. After adjustment at pH 2 with 6M HCl, pepsin (E/S = 1/18 (w/w) was added to initiate the hydrolysis. The pH value was maintained at 2 along the hydrolysis using a pH-Stat with 0.4 M HCl. Samples were taken along the hydrolysis and stored at 4°C in an ice-bath. Hydrolysis was achieved in 180 min of reaction. Samples and hydrolysate were centrifuged 10 min at 5000 rpm, at 4°C and filtered through 0.22 µm filters to remove insoluble particles. All samples were freeze dried before further experiments.
Kjeldahl method was used to evaluate the nitrogen content (N) in the oyster powder.
Quantity of protein (based on nitrogen analysis, N x 6.25) contained in oysters powder was estimated to be 49.5% (w/w).
Grinded oysters powder was of grey-green colour in solution at pH 5.5 (original pH) and went brown after adjustment at pH 2. Solution thickness decreased visually along the hydrolysis and HCl volume addition gave a classical hydrolysis curve (data not shown).
Samples taken along the hydrolysis were tested using the Guinea pig ileum (GPI) test. Electrically induced contractions of Guinea pig ileum were recorded. After addition of an opioid molecule (like enkephalin or morphin), contractions width decrease. The addition of naloxone, a specific agonist of opioid receptors allows the contraction width to increase. The addition of peptic oysters hydrolysate to GPI allowed a decrease of contractions width followed by an increase without the help of naloxone. The effect observed was weak and was not mediated by naloxone. Thus, this effect was not opioid but pseudo-opioid. Oysters peptic hydrolysate seems to contain only few or no potent opioid molecules.

The anti-hypertensive potential of oysters peptic hydrolysate was also examined. The Angiotensin I concerting enzyme (ACE) activity was measured by the method of Holmquist (Holmquist *et al.*,1979). Oysters in water at pH 5.5 did not inhibit the ACE activity whereas after adjustment at pH 2, 859 µg of protein (estimated using micro-Lowry method with BSA as standard (Lowry *et al.*, 1951) contained in oysters solution were able to inhibit 20% of ACE activity. After 20 min of hydrolysis by pepsin, the hydrolysate obtained was able to inhibit almost 90% of the ACE activity (fig. 1). This percentage of inhibition remained stable with samples taken from 20 min to 3 hours of hydrolysis.

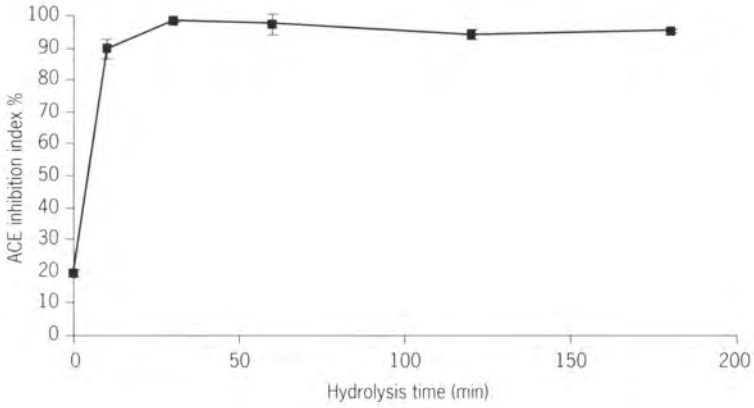

Figure 1
Development of ACE inhibition index of peptic oysters hydrolysate versus hydrolysis time. 859 µg of protein were tested. Mean of 6 tests is presented.

Adjustment of the oysters solution to an acidic pH value probably allowed the release of some molecules able to inhibit ACE. However, hydrolysis of the oysters solution by pepsin was needed to give rise to more or most potent inhibitors of ACE.

From our experiments, the concentration of the 3-hour hydrolysate needed to inhibit 50% of the ACE activity (IC_{50} value) was 72mg/l.

Reverse-phase HPLC analysis of the 3-hour hydrolysate was carried out (fig. 2). About twenty peaks were detected at 215 nm (fig. 2) and only about 13 at 280 nm. Analysis of peaks spectra evidenced few peaks of peptidic nature. The elution profile of the 3 hours hydrolysate was arbitrary divided into 7 fractions which were manually collected and freeze-dried. Each fraction (515 µg of protein) was tested for its ACE inhibitory activities. Results are presented in the table. Except fraction number 1, each other fraction was able to inhibit from 40 to 90% of the ACE activity.

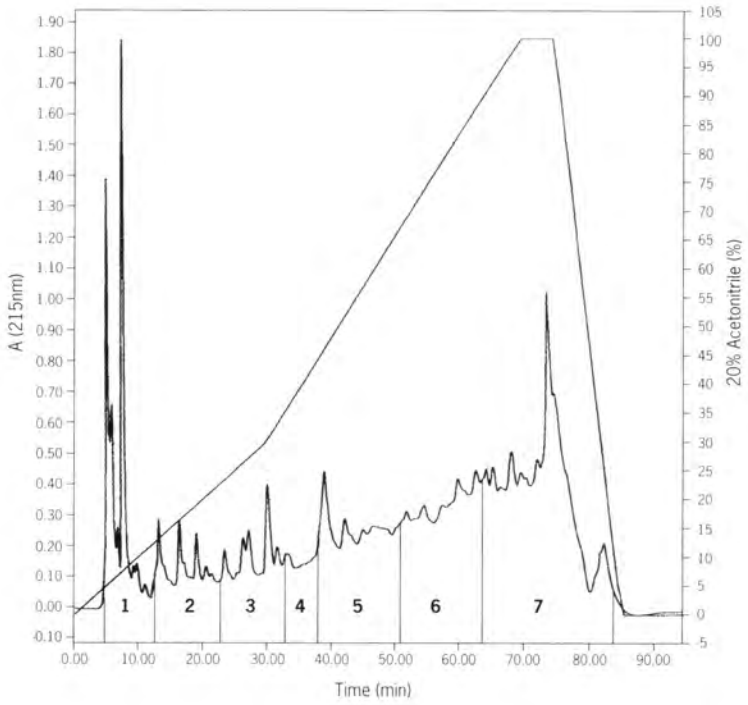

Figure 2
RP-HPLC analysis of 3-hour peptic oysters hydrolysate. 200 mg of hydrolysate powder were applied on the column C18 50 x 300 mm. 20% actetonitrile gradient is also presented. 7 fractions were arbitrary defined.

Biological activities exhibited by oysters peptic hydrolysate (3-hour hydrolysate) and oysters peptic HPLC fractions.

Biological activities		RP-HPLC fraction number							3-hour hydrolysate
		1	2	3	4	5	6	7	
Opioid activity		ND	ND	ND	ND	ND	ND	ND	+/-
ACE inhibition (%)		-	38.5	55.6	62.2	48.3	90.4	55.4	90.4
Anti-oxidant activity		-	-	-	-	-	-	-	ND
Herpes simplex virus inhibition	CC_{50} (µg/ml)	>200	>200	>200	>200	>200	>200	>200	ND
	CE_{50} (µg/ml)	ND	>200	>200	20	1.13	>200	143.6	ND

-: no significant activity, +/-: weak activity (pseudo-opioid effect at high concentrations), ND: not determined.

IC_{50} (concentration of a fraction needed to inhibit 50% of ACE activity) of most interesting fraction in term of ACE inhibition was calculated. Fractions 7, 3 and 6 had IC_{50} of 119, 35.8 and 5.3 mg/l respectively. IC_{50} of fraction 4 was not calculated because a lack of material. Fraction 6 exhibited an IC_{50} roughly 10 times less important than the whole hydrolysate. This fraction was then analysed on RP-HPLC and contained 4 peaks (data not shown). Peaks were collected and tested for their inhibitory activity of ACE. Only 2 peaks contained molecules able to inhibit ACE. They had identical IC_{50}: 16.6 mg/l. This value is 3 times more important than the one obtained for unfractionated frac-

tion 6. This may be due to a synergic effect existing between the molecules contained in fraction 6. Further studies will be undertaken to determine the peptidic sequence of ACE inhibitors in fraction 6.

The anti-oxidant potential of peptic oysters hydrolysate fractions obtained after HPLC fractionation was evaluated using the beta-carotene-linoleate model (Hidalgo *et al.*, 1994). The β-carotene-linoleate bleaching was followed at 470 nm with a spectrophotometer. Presence of anti-oxidant (like vitamin E) prevents β-carotene-linoleate bleaching. Results obtained were not conclusive. No fraction was significantly able to prevent β-carotene-linoleate discoloration. Thus, no anti-oxidant molecules seem to be present in our peptic oysters hydrolysate fractions.

The last biological activity assessed was the antiviral activity of oysters hydrolysate fractions collected after HPLC fractionation. This experiment was done by Cecile Olicard; pH D student. Oysters fractions were added to Vero cells infected by herpes virus type One. After three days incubation, cellular cytotoxicity (CC_{50}), the concentration of fraction needed to kill 50% of cells, and cytopathogenic effect (CE_{50}) were calculated.

Results are presented in the table. No oyster fraction was cytotoxic. More than 200 µg/ml were needed to cause cellular damage. Zovirax, a classical anti-virus drug had an CE_{50} of 0.79 µg/ml whereas two oysters fractions: fraction 4 and fraction 5 had quite similar activities. Further investigations should be undertaken to confirm this last point and identify molecules responsible of this activity.

In conclusion, this study evidenced the presence of potential anti-hypertensive peptides in peptic oyster hydrolysate. The IC_{50} of the most active fraction was of 5.3 mg/l. In order to compare, IC_{50} of captopril, an anti-hyperstensive drug was 3.02 µg/l in the same experimental conditions. The IC_{50} of a milk derived peptide alpha-lactorphin, well-known as an inhibitor of ACE, was 382 mg/l (unpublished results). Thus, the oyster peptic hydrolysate fraction obtained by RP-HPLC seems of course less potent than a chemical drug but more potent than a pure peptide issued from milk protein. Moreover, since the enzyme used, pepsin, is a gastric enzyme, it would mean that potential inhibitors of the ACE could be produced after oyster digestion in the organism. Proteolysis of whole oysters seems to be an interesting biotechnological transformation process since bioactive molecules could be obtained quickly. In our case, maximal inhibition was obtained after only 20 min of hydrolysis. A careful control of the hydrolysis process would lead to the obtention of interesting hydrolysis products readily available. After identification of peptides responsible of the inhibition of ACE, further tests *in vivo* should be perform in order to assess the activity of identical synthetic molecules. Further investigations should evidenced if oyster hydrolysate contains anti-*Herpes simplex* virus molecules. Others investigations should evidenced if oyster could be used as health-care food in the growing nutraceutical market.

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Microbial nitrogenous substrate from protein hydrolysate of non-tradable oysters, a marine equivalent to yeast extract?

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Abstract

The flesh of non-tradable (over sized) oysters was submitted to an enzymatic hydrolysis in order to produce a microbial nitrogenous substrate. Oyster is an interesting raw material as it can also be considered as a source of minerals (calcium, iron, copper, magnesium, silicon, phosphorus, sodium, manganese for a total of over a gram per 100 g), lipids (1.5%), carbohydrates (5%) and vitamins (C, PP, B group, A). Different enzyme (Alcalase 2.4 l from Novozymes) / substrate ratios were tested and the protein hydrolysates investigated (N solubilisation, peptide profiles...). In conditions where oligopeptides and amino acids are predominant (E/S 0.05, pH 8, 50°C, 3 hrs), the final yield, including the clarification step, based on N release, was about 60%.
The oyster hydrolysate was then introduced in microbial culture media, in a similar way of yeast extract, i.e. complement of another nitrogenous substrate.
Oyster nitrogenous complement added to a casein (Bactotryptone from Difco) or a tuna peptone (produced in our laboratory) gave an improvement of the microbial biomass production for two test strains *Saccharomyces cerevisiae* and *Lactobacillus casei*.

Introduction

Seafood products are obtained from a wide variety of animal species. However, only a portion of those animals is usually consumed as food. The remainder is a by-product, often high in protein, that can be processed into useful products (fish protein concentrate, fish meal, fish silage, animal feed...). Additionally, when harvesting fish, many species that are not used as human food are also caught. The whole of these "trash"-fish can consequently be processed into the same useful products (Diniz & Martin, 1997).

The proteinaceous material from fish can be hydrolysed by chemicals or enzymes. Comparison of Fish protein hydrolysates (FPH) can be based upon various characteristics (total nitrogen content, soluble nitrogen content, amino acids composition, peptide profile...). FPH could be used in biotechnology as nitrogenous substrates (Dufossé *et al.*, 2001), these products are named peptones.

Peptones are defined as protein hydrolysates that are soluble in water and not heat coagulable (Green *et al.*, 1977). These products may have significant value for the fisheries industries as their market prices are quite higher than usual by-products such as fish silage, fish meal...

Growth substrates costs make often the major part of the production cost of microbial cells and bioproducts from the fermentation industry (de La Broise *et al.*, 1998). The nitrogen source is usually the most expensive component of bacterial growth substrates, and at present, it is obtained from plants, dairy proteins such as casein or whey, and slaughterhouse waste. Up today the fish protein hydrolysates have been investigated only to a minor extent, and their uses in industrial processes are still poor. Hydrolysates from fish produced by means such as autolysis or enzymic proteolysis have been obtained from 34 species of fish. Among these, 17 were tested for microbial growth (Dufossé *et al.*, 1997; review). Except these researches on fish protein, to our knowledge, no data has been published on shellfish upgrading for microbiological purposes.

As we started a research project on the production of oyster flesh protein hydrolysate in our laboratory, we intended to include this hydrolysate from *Crassostrea gigas* in microbial culture media.

In this paper, our oyster protein hydrolysate was incorporated in a similar way of yeast extract, i.e. complement of another nitrogenous substrate, e.g. usual biotechnological casein one (Bactotryptone from Difco) or lab-made tuna peptone (Guérard *et al.*, 2001a). Combined with a fish peptone, the oyster hydrolysate could offer a 100% marine alternative for microorganism cultivation.

As methodologies applied up to now are quite inadequate in peptone assessment, we used an evaluation method based on Gompertz modelling of microbial growth.

To demonstrate the general interest of such nitrogenous substrates for the biotechnology industry, 2 microorganisms were tested:

1) a gram positive bacteria, *Lactobacillus casei*, which is quite hard to grow, is present in dairy starters, and is also used for lactic acid production or post-koji making;

2) a common yeast, *Saccharomyces cerevisiae*, spread all over the biotechnology field and the food manufacture.

Growths of these microorganisms were investigated using optical density measurement and modelling the growth curves gave access to the kinetic parameters, i.e. lag phase (hrs) and maximum growth rate (h^{-1}). Biomass value (A_{max} 650 nm) represents the third parameter followed in this study.

Material and methods

Preparation and characterisation of the oyster protein hydrolysate

Freeze-dried oyster flesh was a kind gift from Dr Philippe Roy, Ifremer Nantes, France. Alcalase 2.41 was provided by Novo Nordisk (Denmark). Hydrolysis experiments were carried out in a 1-liter reactor using the pH-stat method in controlled hydrolysis conditions (pH, temperature, enzyme concentration and stirring speed). Determination of the degree of hydrolysis (DH) was done according to Adler-Nissen (1982). The molecular weight distribution of peptides was analysed using size exclusion chromatography (SEC) using a Superdex Peptide HR 10/30 column; for running condition refer to Guérard *et al.* (2001b).

Microorganisms and cultivation media

The bacteria *Lactobacillus casei* (ATCC 7469), and the yeast *Saccharomyces cerevisiae* (IUT Quimper) were grown at 25°C in liquid media previously autoclaved at 121°C for 15 minutes. Bacteria (= B) and yeast (= Y) media consisted in (w/w): B = 2% Y = 8% glucose, 0.5% peptone, 0 to 0.1% oyster hydrolysate, 0.35% KH_2PO_4, 0.15% K_2HPO_4, 0.1% NaCl, 0.2% $MgSO_4$, 7 H_2O, pH 6.0. Cultivation was performed during 3 to 5 days on a rotatory shaker (150 rpm) in 250 ml culture flasks containing 100 ml of medium.

Growth kinetics, modelling the growth curve

Bacterial growths were followed every 2 hours using optical density measurements (650 nm). Each growth curve presented hereafter for a microorganism/peptone combination was obtained from 4 individual cultures.

A lot of mathematical models can be used to describe lag phase (λ), maximum growth rate (μ_{max}), and maximum biomass at the stationary phase (A) (Zwietering *et al.*, 1990). Gompertz model (1), well suited for such a purpose, was applied to the growth curves obtained on peptones.

$$\log \frac{N}{N_0} = A * \exp\left(-\exp\left(\frac{\mu_{max} * \exp(1) * (\lambda - t)}{A} + 1\right)\right)$$

N_0 = initial population; N = population at instant t

Calculations were run on Excel (Microsoft) using the least square method to adjust the model to the data, and the correlation coefficient to estimate the fitness of this adjustment.

Results and discussion

Preparation of the oyster protein hydrolysate

Crassostrea gigas flesh from the Atlantic Ocean was not heat inactivated prior to our experiments, so endogenous enzymes were able to hydrolyse the proteic material: DH value of 3.2% after 3 hours of incubation and nitrogen release (NR), i.e. nitrogen solubilisation, of 45.6% (fig.1, "no added enzyme" curve). This choice of non heat inactivation was made in order to keep intrinsic properties of the oyster starting material (heat sensitive vitamins, loss of minerals...).

Following addition of Alcalase 2.4 l, optimum hydrolysis was obtained at a 5% prot/prot E/S ratio (5 g of enzymatic proteins added to 95 g of oyster proteins): nitrogen release up to 96% (fig. 1).

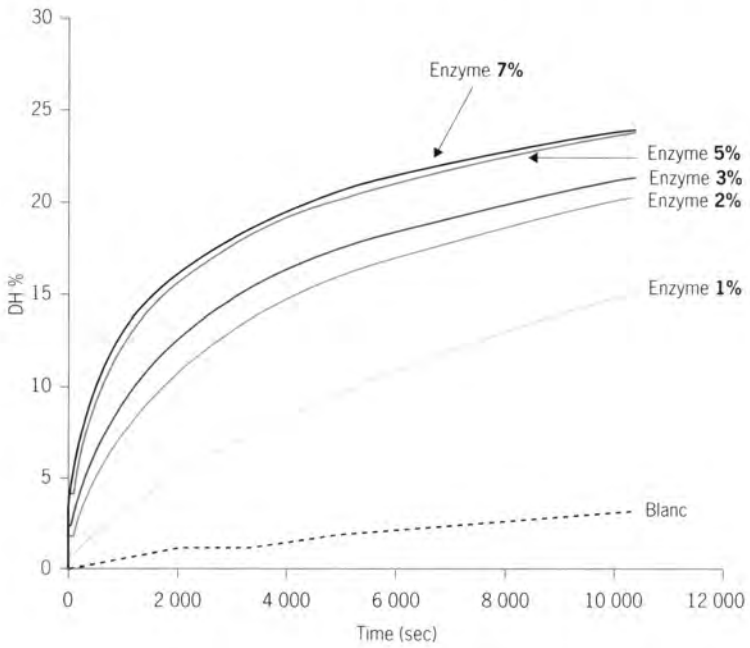

Figure 1
Hydrolytic curves for oyster flesh treated with Alcalase. Various enzyme concentrations (% prot/prot, liquid enzymatic preparation/flesh). Experimental conditions: T 50°C, pH 8, 3 hrs running time.

A pilot scale production of oyster hydrolysate was then undertaken:
1) hydrolysis with added protease;
2) centrifugation of the hydrolysis medium in order to reject residues;
3) freeze-drying of the supernatant.

Characterisation of the oyster protein hydrolysate

SEC chromatogramms: the enzymatic treatment of oyster flesh by Alcalase produced major fractions with molecular weights ranging from 6500 Da to free amino acids. An increase of the low molecular weight fractions (< 500 Da) was noticed with hydrolysis time or higher enzyme/substrate ratios.

Typical SEC chromatogramms are shown in figure 2. Similar patterns were obtained for tuna and casein peptone (named Bactotryptone™), except that the latter showed specific narrow peaks at 37 and 45 min retention times. Oyster protein hydrolysate (OPH) presented a profile more directed towards small sized peptides.

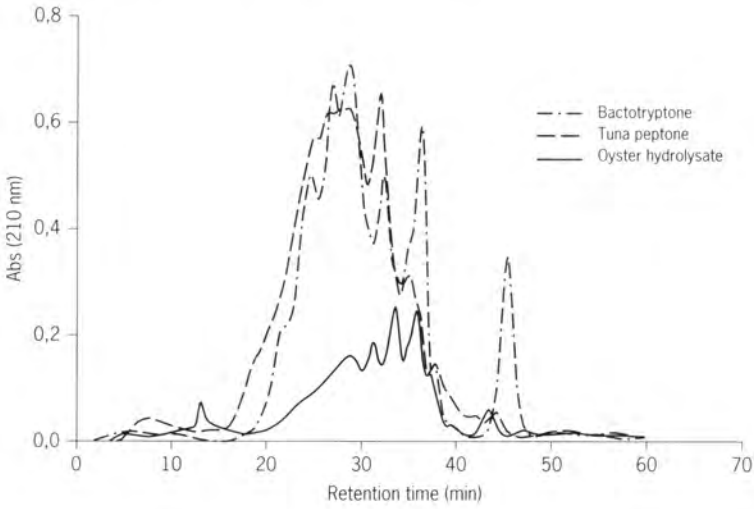

Figure 2
Size exclusion chromatogramms of the nitrogenous products used as bacterial substrates.

Amino acid compositions of oyster flesh and OPH indicate a quite good conservation of the amino acids in the final product, except for cystein that was destroyed during hydrolysis.

Amino acid compositions of the oyster (OYS) flesh (starting material) and the resulting product, oyster protein hydrolysate (OPH).

AA	*Asp*	*Ser*	*Glu*	*Gly*	*His*	*Arg + Thr*	*Ala*	*Pro*	*Cys*	*Tyr*	*Val*	*Met*	*Lys*	*Ileu*	*Leu*	*Phe*
OYS	3.03	1.80	4.97	2.68	1.10	8.24	2.16	3.16	0.57	1.34	1.90	0.93	2.52	1.82	3.13	3.31
OPH	3.03	1.85	5.07	2.87	0.89	14.9	2.18	2.91	0	1.51	2.08	1.03	2.11	1.88	3.14	3.41

Microbial growth

Oyster protein hydrolysate (OPH) was introduced (0 to 0.1%) in liquid culture media containing a main nitrogenous substrate, casein or tuna peptone (0.5%). Biomass production was followed in order to know if OPH gave improvement of the kinetics over the simple "added N effect", i.e. more biomass simply related to more nitrogen.

As shown in figure 3, addition of OPH in *Lactobacillus casei* cultures increased biomass level in such an extent that added nitrogen could not entirely explain the phenomenon. Moreover, in the same experimental conditions, the production of *Saccharomyces cerevisiae* biomass was increased by 57.2% (calculations based on g dry matter/g added nitrogen).

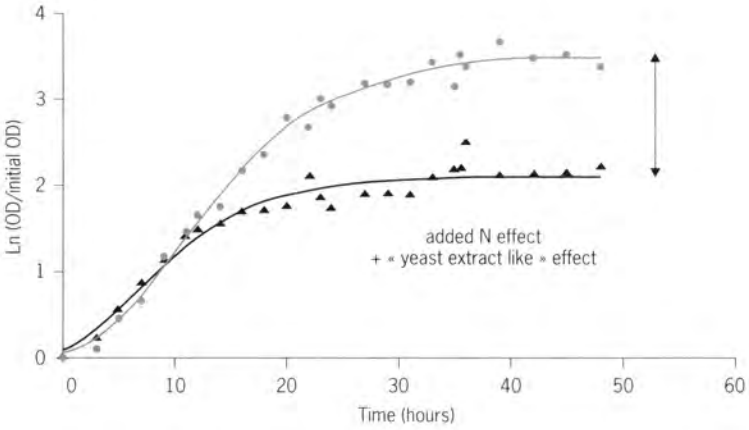

Figure 3
Growth of *Lactobacillus casei* in liquid media containing casein peptone as sole nitrogen source (▲) or casein peptone supplemented with oyster protein hydrolysate (●). [solid line = adjustment of the model].

Our work demonstrate that oyster protein hydrolysate has a "yeast like effect" that should be investigated more deeply (role of B vitamins, minerals...).

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The glue of sea cucumber Cuvierian tubules: a novel marine bioadhesive

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Abstract

Several species of sea cucumbers possess a very peculiar and specialized defence system: the so-called Cuvierian tubules. It is mobilized when the animal is mechanically stimulated, resulting in the discharge of a few white filaments, the tubules. In seawater, the expelled tubules considerably lengthen and become sticky upon contact to any object. The great adhesivity of the outer epithelium combined with the high tenacity of the collagenous core makes Cuvierian tubules very efficient at entangling and immobilizing most potential predators. The fact that Cuvierian tubules are able to form strong adhesive bonds in a few seconds to a variety of substrata suggests that their glue could offer novel features or performance characteristics for applications as underwater adhesives. This paper compares some recent data on the mechanical and biochemical properties of the adhesive from holothuroid Cuvierian tubules with the properties of the adhesives from other marine invertebrates. The adhesive of Cuvierian tubules stands apart from every other marine invertebrate adhesive described so far by its gross biochemical composition as well as by the amino acid composition of its protein fraction. Yet, the adhesive strength of this organ falls within the range of tenacities measured for other adhesive systems. Cuvierian tubules could therefore be an interesting model system in the search for a novel bioadhesive with biotechnological applications.

Introduction

Adhesion (attachment with adhesive substances) is a way of life in the sea. Indeed, representatives of bacteria, protoctists (including macroalgae), and all animal phyla living in the sea attach to surfaces, including other organisms (Walker, 1987). Adhesion is particularly developed and diversified in invertebrates. It is involved in various functions such as the attachment of the larvae and/or the adults to the substratum, the locomotion, the taking of food, or the building of tubes or burrows (Walker, 1987; Tyler, 1988; Flammang, 1996). Adhesion may be permanent as in sessile invertebrates that cement themselves to the substratum (e.g., barnacles), or non-permanent as in those benthic organisms that

move around at some times and attach themselves strongly but temporarily to the substratum at other times (e.g., limpets) (Walker, 1987; Tyler, 1988; Flammang, 1996).

The fact that marine invertebrates produce adhesives that act in the presence of water has aroused increasing scientific and technological attention because such adhesives are sorely needed for applications in underwater construction or in the medical and dental fields (Strausberg & Link, 1990; Yamamoto, 1995; Taylor & Waite, 1997). Most studies of invertebrate adhesive systems have focused on the characterization of the permanent adhesives from sessile organisms such as mussels, barnacles or tube-dwelling worms (see, e.g., Naldrett & Kaplan, 1997; Taylor & Waite, 1997; Kamino *et al.*, 2000). This is because in these organisms the adhesives are secreted as a fluid and then gradually solidify to form a cement possessing high adhesive and cohesive strengths (Walker, 1987). However, the adhesive biochemistry of sessile invertebrates has proven to be quite complex: not only do the adhesives consist of a blend of several different proteins (Taylor & Waite, 1997; Kamino *et al.*, 2000; Waite & Qin, 2001) but their hardening relies on enzymatic modifications of the secreted proteins (Dougherty, 1997; Hansen *et al.*, 1998). So far, non-permanent adhesives have attracted much less interest and the best characterized is that from the limpets. In these organisms, the adhesive is a hydrogel comprising high molecular weight polysaccharides and low molecular weight proteins associated in a non-covalently linked complex (Grenon & Walker, 1980; Smith *et al.*, 1999a).

In the search for simpler models of bioadhesion, other adhesive systems from marine invertebrates are therefore also worth investigating. One of these systems is the defensive reaction occurring in some species of holothuroid echinoderms (sea cucumbers), and which involves the Cuvierian tubules (Flammang, 1996; Hamel & Mercier 2000). Several species of holothuroids, all belonging exclusively to the family Holothuriidae, possess this very peculiar and specialized defence system. It is mobilized when the animal is mechanically stimulated, resulting in the discharge of a few white filaments, the tubules. In seawater, the expelled tubules lengthen considerably and become sticky upon contact with any object (VandenSpiegel & Jangoux, 1987). The great adhesivity of the outer tubule epithelium combined with the high tensile strength of their collagenous core make Cuvierian tubules very efficient for entangling and immobilizing most potential predators (VandenSpiegel & Jangoux, 1987; Hamel & Mercier, 2000).

This paper compares some recent data on the mechanical and biochemical properties of the adhesive from holothuroid Cuvierian tubules with the properties of the adhesives from other marine invertebrates. Its aim is to find out if Cuvierian tubule glue could offer novel features or performance characteristics for applications as an underwater adhesive.

Adhesive strength

The evaluation of the adhesive strength in marine invertebrates is usually done by measuring their tenacity, which is the adhesion force per unit area and is expressed in Pascals (Pa). According to the taxonomic group considered, tenacities of marine organisms range from about 1 to 2000 kPa (see, e.g., Walker, 1987, for review). However, many studies have shown that several factors may profoundly influence the tenacity of invertebrates (see, e.g., Grenon & Walker, 1981). For example, the physical (e.g., roughness) as well as chemical characteristics (e.g., hydrophobicity, surface charges) of the substratum are known to change the tenacity of organisms by up to an order of magnitude (Young & Crisp, 1982; Yule & Walker, 1987). Adhesion force measurements can also be performed using either a pull at right angle to the substratum (normal), a pull parallel to the substratum (shear), or a combination of both. It was demonstrated that the direction of pull also strongly influences the measured tenacity of marine invertebrates (Grenon & Walker, 1981). As a consequence, great care should be exercised when comparing values of tenacity extracted from different studies. Table 1 presents the tenacities of various marine invertebrates, all measured on smooth glass and by pulling the organism or its adhesive organ normal to the substratum. Comparison of the values indicates that organisms attaching permanently show a higher tenacity than organisms attaching temporarily.

Cuvierian tubule adhesive strength on glass has been measured in seven species of sea cucumbers belonging to the genera *Bohadschia, Holothuria* and *Pearsonothuria* (Flammang *et al.*, unpubl. obs.). The mean normal tenacities observed varied from about 30 to 135 kPa. These tenacities fall within the range of adhesive strengths described for marine organisms (tab. 1). They lie, however, among the lowest values observed, being closer to the tenacity of organisms using non-permanent adhesion than to those of sessile marine invertebrates using permanent adhesion. In natural conditions, however, Cuvierian tubule adhesion functions under shear loading and not under orthogonal loading. The shear tenacity of Cuvierian tubules can be al least four times higher than their normal tenacity. For example, the normal tenacity of the tubules of *Holothuria forskali* on paraffin wax is about 4 kPa (Flammang *et al.*, unpubl. obs.) whereas shear tenacity on the same substratum is about 15 kPa (Zahn *et al.*, 1973). This is because in the case of a cylindrical structure like a tubule, a pull normal to the substratum induces extensive peeling during detachment. On the other hand, peeling does not occur when Cuvierian tubules are detached in shear (Zahn *et al.*, 1973). It is well-known that peeling dramatically reduces adhesion in marine organisms. For example, in the sea anemone *Actinia equina*, Young *et al.* (1988) increased the measured normal adhesive strength on Tufnol (a plastic) from 20 kPa to 460 kPa by just switching to an experimental design that reduced the incidence of peel. This means that the

tenacities of sea cucumber Cuvierian tubules presented in table 1 are probably underestimates and suggests that Cuvierian tubules compare well with adhesive systems of other marine invertebrates in terms of adhesive strength.

Table 1 - Comparison of the mean normal tenacity of some marine invertebrates on smooth glass. When two values are indicated, they represent minimum and maximum means from different species, except in *Mytilus edulis* for which they represent measurements made at different seasons.

Taxa	Species	Structure involved in adhesion	Tenacity (kPa)	Reference
Permanent adhesion				
Mollusca Bivalvia	*Mytilus edulis* (February-June)	Byssus	316-750	Young & Crisp, 1982
Crustacea Cirripedia (adult)	*Balanus crenatus*, *Semibalanus balanoides*	Base	230-520	Yule & Walker, 1987
Non-permanent adhesion				
Cnidaria Anthozoa	*Actinia equina*, *Metridium senile*	Foot	19-43	Young *et al.*, 1988
Mollusca Gasteropoda	*Patella vulgata*	Foot	228	Grenon & Walker, 1981
Crustacea Cirripedia (cyprid larva)	*Semibalanus balanoides*	Antennules	98	Yule & Walker, 1987
Echinodermata Asteroidea	*Asterias rubens*, *Asterias vulgaris*	Tube feet	170-198	Paine, 1926; Flammang & Walker, 1997
Cuvierian tubule adhesion				
Echinodermata Holothuroidea	*Bohadschia marmorata*, *Bohadschia subrubra*, *Holothuria forskali*, *Holothuria impatiens*, *Holothuria leucospilota*, *Holothuria maculosa*, *Pearsonothuria graeffei*	Cuvierian tubules	30-134	Flammang *et al.*, unpubl. obs.

Composition of the adhesive

In marine invertebrates, adhesive secretions are always predominantly made up of proteins. Yet, their biochemical composition varies from one taxonomic group to another (Flammang *et al.*, 1998; Smith *et al.*, 1999a). As a general rule, permanent adhesives consist almost exclusively of proteins: for example, mussel byssal plaques contain about 99% proteins (Cook, 1970) and barnacle cement about 85% (Walker, 1972). On the other hand, non-permanent adhesives are made up of an association of proteins and carbohydrates, usually in a 2:1 ratio and that together represents about 50% of the adhesive dry weight (see Whittington & Cribb, 2001, for review). The carbohydrate fraction is mostly in the form of acidic and sulphated sugars and associated to an important inorganic fraction accounting for the other 50% of the adhesive material (Grenon & Walker, 1980; Flammang *et al.*, 1998; Smith

et al., 1999a). The Cuvierian tubule adhesive is made up essentially of organic material (about 90%), the inorganic residue amounting only to about 10% (De Moor *et al.*, 2002). The organic fraction comprises both proteins and carbohydrates in a 3:2 ratio. The composition of the Cuvierian tubule adhesive is therefore reminiscent of non-permanent adhesives by its association of proteins and carbohydrates. However, it differs from them by the fact that the carbohydrate fraction is in the form of neutral sugars and not acidic sugars, and by the fact that the inorganic fraction is much smaller. Cuvierian tubule adhesive thus appears as unique among invertebrate adhesives in terms of its gross biochemical composition.

As far as the amino acid composition of the protein fraction is concerned, all the marine bioadhesives characterized so far have in common their richness in small side-chain amino acids as well as in charged and polar amino acids. These characteristics were indeed observed in flatworms (Hamwood *et al.*, 2002), mussels (Benedict & Waite, 1986; Waite *et al.*, 1989), limpets (Grenon & Walker, 1980; Smith *et al.*, 1999a), tubeworms (Jensen & Morse, 1988), barnacles (Walker, 1972; Kamino *et al.*, 1996; Naldrett & Kaplan, 1997), sea stars (Flammang *et al.*, 1998), and sea cucumbers (De Moor *et al.*, 2002). Charged and polar amino acids are probably involved in adhesive interactions with the substratum through hydrogen and ionic bonding (Waite, 1987). Small side-chain amino acids, on the other hand, are often found in large quantities in elastomeric proteins (Tatham & Shewry, 2000). These proteins are able to withstand significant deformations without rupture before returning to their original state when the stress is removed (Smith *et al.*, 1999b). Marine glues thus appear to be tailored for both high adhesive strength and high cohesive strength.

Despite these similarities, the composition of marine invertebrate adhesives is variable from one species to another. To quantify this variability, the method of Marchalonis and Weltman (1971) was used. It allows the determination of relatedness among proteins based upon statistical analysis of differences in their amino acid composition. A parameter called $S\Delta Q$ is calculated by pairwise comparison of the percentages of each amino acid constituting the proteins. Marchalonis and Weltman (1971) reported that values of $S\Delta Q \leq 100$ indicate relatedness. Here, this method has been extended to whole adhesives, which are usually blends of different proteins, based on the assumption that if they enclose closely related proteins their whole amino acid compositions will be similar too. The values of $S\Delta Q$ for comparisons between the adhesives of twelve invertebrate species belonging to seven taxonomic groups are given in table 2. Three amino acids (i.e., half-cystine, hydroxyproline and di-hydroxyphenylalanine [DOPA]) that were not considered by Marchalonis & Weltman (1971) have been taken into account because they are important constituents of some marine adhesives (Taylor & Waite, 1997; Kamino *et al.*, 2000). Aspartic acid and asparagine, glu-

tamic acid and glutamine were taken as Asx and Glx respectively as in the original method. The level of significance was also set at 100, but two values just above than 100 were also considered as indicating relatedness (tab. 2). Values given in table 2 show that the adhesives of every species within a same taxonomic group are related suggesting, as expected, that they are homologous. More interesting is the relationship between the adhesives of all the species using non-permanent adhesion, despite the fact that they belong to very disparate phyla (i.e., platyhelminthes, molluscs and echinoderms; table 2). This relationship indicates convergence in composition because of common function and selective pressures. On the other hand, such an analogy is not observed for the adhesives of sessile invertebrates using permanent adhesion. Indeed, the adhesives from mussels, tubeworms and barnacles differ one from another (tab. 2). The protein fractions of mussel byssal plaque and polychaete cement have in common the presence of DOPA in their composition (Benedict & Waite, 1986; Jensen & Morse, 1988; Waite *et al.*, 1989). This unusual amino acid is involved in surface coupling (adhesion) and cross-linking (cohesion) of these adhesives (Taylor & Waite, 1997). However, the tubeworm adhesive stands apart from any other adhesive by its very high content of serine (Jensen & Morse, 1988). Barnacle cement, on the other hand, contains no DOPA and appears to be closer to non-permanent adhesives (tab. 2). They have in common the importance of disulphide bonds in their cross-linking (Flammang *et al.*, 1998; Smith *et al.*, 1999a; Kamino *et al.*, 2000; De Moor *et al.*, 2002). It was already suggested that barnacle adhesive would function as a highly viscous fluid and not as a true solid cement (see Yule & Walker, 1987, for review). This hypothesis was based on the fact that juvenile barnacles can move at least 2 cm under the pressure of neighbours. It is further corroborated by the similarity of the composition of the protein fraction from barnacle adhesive with that of viscous non-permanent adhesives. As for the adhesive from holothuroid Cuvierian tubules, it differs from every other marine bioadhesive by its amino acid composition (tab. 2). The protein fraction of this adhesive is particularly rich in glycine (De Moor *et al.*, 2002), resembling in this way mussel adhesives (Benedict & Waite, 1986; Waite *et al.*, 1989).

Table 2 - Values of SΔQ for comparison among adhesives in marine invertebrates. Values in bold indicate relatedness; boxes frame species belonging to a same taxonomic group (i.e., mussels, barnacles and limpets, respectively). CT, Cuvierian tubule adhesion.

	Permanent adhesion							Non-permanent adhesion				CT
	Gd	Me	Pc	Bc	Be	Bh	Mr	Es	Ll	Pv	Ar	Hf
Gd	0	-	-	-	-	-	-	-	-	-	-	-
Me	90	0	-	-	-	-	-	-	-	-	-	-
Pc	714	706	0	-	-	-	-	-	-	-	-	-
Bc	191	348	989	0	-	-	-	-	-	-	-	-
Be	263	430	1038	107	0	-	-	-	-	-	-	-
Bh	216	383	888	97	97	0	-	-	-	-	-	-
Mr	160	364	907	72	76	64	0	-	-	-	-	-
Es	124	357	971	186	222	195	78	0	-	-	-	-
Ll	214	480	1130	151	213	139	77	105	0	-	-	-
Pv	176	385	1093	99	128	93	39	96	60	0	-	-
Ar	116	323	1007	74	148	82	34	62	34	32	0	-
Hf	145	131	818	433	580	457	427	345	445	406	345	0

Mussels: Gd, *Geukensia demissa*, Waite *et al.*, 1989; Me, *Mytilus edulis*, Benedict & Waite, 1986. Tube-dwelling worm: Pc, *Phragmatopoma californica*, Jensen & Morse, 1988. Barnacles: Bc, *Balanus crenatus*, Walker, 1972; Be, *Balanus eburneus*, Naldrett & Kaplan, 1997; Bh, *Balanus hameri*, Walker, 1972; Mr, *Megabalanus rosa*, Kamino *et al.*, 1996. Flatworm: Es, *Entobdella soleae*, Hamwood *et al.*, 2002. Limpets: Ll, *Lottia limatula*, Smith *et al.*, 1999a; Pv, *Patella vulgata*, Grenon & Walker, 1980. Sea star: Ar, *Asterias rubens*, Flammang *et al.*, 1998. Sea cucumber: Hf, *Holothuria forskali*, De Moor *et al.*, 2002.

Conclusion and prospects

The best-characterized marine bioadhesive is that from the blue mussel, *Mytilus edulis*. In this species, several proteins have been identified and characterized that co-occur as a complex blend in the byssal adhesion plaques (Waite, 1992; Taylor & Waite, 1997; Waite & Qin, 2001). So far, however, only one of these proteins (Mefp-1) has been used in biotechnological applications. Several imaginative applications have been developed for this protein (or for derived peptides), including, among others: tissue adhesives as sealants for medical, surgical and dental applications; enzyme, cell, and tissue-immobilizing agents; anticorrosives and metal scavengers (Burzio *et al.*, 1997; Taylor & Waite, 1997). However, several limitations have come out such as the requirement of post-translational modifications to certain amino acids, or the need for a separate enzyme for curing (Strausberg & Link, 1990). New models of bioadhesion are therefore sought that could overcome these problems.

The adhesive of sea cucumber Cuvierian tubules stands apart from every other marine invertebrate adhesive described so far by its gross biochemical composition as well as by the amino acid composition of its protein fraction. Yet, the adhesive strength of this organ falls within the range of tenacities measured for other adhesive systems. Moreover, it possesses some interesting characteristics like its capacity to form adhesive bonds in a matter of seconds (less than 10 s; Zahn *et al.*, 1973).

Cuvierian tubule adhesive could therefore offer novel features or performance characteristics for biotechnological applications. Work is currently in progress to identify, purify and characterize the constitutive proteins of Cuvierian tubule adhesive. The complete elucidation of their structure and physico-chemical characteristics is an obligatory prerequisite before any application can be envisaged.

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Marine biotechnology : an overview of leading fields

The IX^th^ meeting of the European Society for Marine Biotechnology held on 12-14 May 2002 in Nantes was an opportunity to make assessments on marine biotechnology leading fields and meet both researchers and industrials. Communications reported here was focused on bioactive compounds from marine organisms and plants (proteins, peptides, enzymes, lipids, polysaccharides...) and their potential uses in various fields as human or animal nutrition (aquaculture, microbiology) and human health (pharmacy). A special session was dedicated to marine polysaccharides from algae and bacteria, accurate examples on their potential uses as new therapeutic agents have been displayed. The biodiversity of marine resources offers many applications in industrial field and contributes to the expansion of the marine biotechnology.

Key words : marine organism, bioactive compound, aquaculture, biopolymer, enzyme, protein, peptide.

Biotechnologie marine : panorama des principaux domaines

Le IX^e^ colloque de la Société européenne pour la biotechnologie marine s'est tenu du 12 au 14 mai 2002 à Nantes, il a permis de réunir en un même lieu les acteurs du monde de la recherche et ceux de l'industrie afin d'exposer et de débattre des avancées dans les principaux domaines de la biotechnologie marine. La majorité des interventions a porté sur les molécules bioactives issues d'organismes et de végétaux marins en donnant des informations sur leur nature (protéine, peptide, enzyme, lipide, polysaccharide...) et sur leurs utilisations potentielles dans des domaines aussi divers que la nutrition humaine et animale (aquaculture, microbiologie) et la santé humaine (pharmacie). Une session a même été totalement consacrée aux polysaccharides marins issus d'algues et de bactéries, des exemples précis sur leur potentiel en santé humaine ont été donnés. Il ressort de ce congrès que la biodiversité des ressources marines offre de nombreux débouchés dans l'industrie et contribue à l'essor de la biotechnologie marine.

Mots-clés : organisme marin, molécule bioactive, aquaculture, biopolymère, enzyme, protéine, peptide.

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