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Proteolysis of shrimp by-products (*Penaeus monodon*) from Madagascar

Proteólisis de derivados de langostino (*Penaeus monodon*) de Madagascar

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Abstract :

To generate and recover elements from shrimp heads, proteolysis was performed with commercial acidic or alkaline proteases for 22 h. The resulting phases were characterized for protein and lipid content and amino acid composition while the molecular profiles of soluble peptides were established. Whatever the protease used, more than the half of the initial dry matter was found into aqueous phase including most of the proteins while lipids were found to remain insoluble. Hydrolysates were mainly constituted by small peptides (>80% below 1000 Da) with up to 14.15 amino acids identified. Moreover, all the hydrolyses have led to an increase of the amount of essential amino acids into the hydrolysates, including lysine. Thus, such proteolysis of shrimp heads can increase their potential nutritional value (small peptides with high content in essential amino acids) while allowing the concentration of lipids into insoluble phase and a partial demineralisation of the exoskeleton.

Para crear y recuperar elementos de las cabezas de langostino se llevó a cabo una proteólisis con proteasas ácidas y alcalinas comerciales durante 22 horas. Las fases resultantes se caracterizaron por contenido proteico y lipídico, composición de aminoácidos, mientras los perfiles moleculares de péptidos solubles fueron establecidos. Cualquiera fuese la proteólisis, más de la mitad de la materia seca inicial se encontró en fase acuosa, incluida la mayor parte de las proteínas, mientras los lípidos resultaron mantenerse insolubles. Los hidrolizatos estuvieron constituidos principalmente por pequeños péptidos (>80% por debajo 1000 Da) con hasta 14.15 aminoácidos identificados. Es más, todas las hidrólisis llevaron a un incremento de la cantidad de aminoácidos esenciales en los hidrolizatos, incluido lisina. Así, dicha proteólisis de cabezas de langostino puede incrementar de este modo su valor nutricional potencial (pequeños péptidos con un alto contenido en aminoácidos esenciales) mientras permite la concentración de lípidos en fase soluble y una desmineralización parcial de esqueleto externo.

Keywords : by-products ; shrimp; proteolysis ; hydrolysate

Palabras clave : derivados ; langostino; proteólisis ; hidrolizado

1. Introduction

Worldwide fishery production was reported to be about 144 million tonnes in 2006, 36% of which was from aquaculture, and approximately 33% are used for non-food purpose. Shrimp constitutes the main fish traded in value term with 16% of the total value of internationally traded fishery products (Food and Agriculture Organization [FAO], 2009).

In Madagascar, shrimp exportation constitutes 73% of the total fisheries product. The average of production is 12,977 tonnes in 2006, including 51% of contribution from cultivated shrimp (Mission Economique de Tananarive, 2008).

Shrimp transformation lead to wastes generation. In Madagascar, they are composted to fertilizer or used in animal feeding; industrial transformation contributes only to about 10% of those products. These wastes are mainly constituted by cephalothorax and exoskeleton which represent at least 40% of the whole shrimp. Many studies have revealed that they contain useful components like proteins, lipids, astaxanthin and of course chitin, which is well known as a marketable product (Heu *et al.*, 2003). Indeed, numerous works have been conducted for recovering those valuable products. Chemical procedures were developed to extract chitin and chitosan (Sagheer *et al.*, 2009) and astaxanthin (Sachindra & Mahendrakar, 2005; Sachindra, Bhaskar & Mahendrakar, 2006). Fermentation and silage were experienced to recover chitin (Beaney, Lizardi-Mendoza & Healy, 2005; Bhaskar, Suresh, Sakhare & Sachindra, 2007; Rao, Munoz & Stevens, 2000), astaxanthin (Cira, Huerta, Hall & Shirai, 2002; Fagbenro & Bello-Olusoji, 1997; M. Healy, Green & A Healy, 2003) but also for animal feeding (Oliveira Cavalheiro, Oliveira de Souza & Bora, 2007). Enzymatic hydrolysis was used for chitin extraction (Ferrer *et al.*, 1996; Gagné & Simpson, 1993; Gildberg & Stenberg, 2001; Synowiecki & Al-Khateeb, 2000), for carotenoprotein recovering (Babu, Chakrabarti & Surya Sambasivarao, 2008; Simpson & Haard, 1985), and for generation of bioactive or functional peptides (He, Chen, Sun, Zhang & Gao, 2006; Ravallec-Plé & Van Wormhoudt, 2003; Ruttanapornvareesakul *et al.*, 2005; Ruttanapornvareesakul *et al.*, 2006).

Proteins represent the major component of shrimp heads (Fagbenro & Bello-Olusoji, 1997; Cira *et al.*, 2002; Ruttanapornvareesakul *et al.*, 2005; Coward-Kelly, Agbogbo & Holtzapple, 2006; Babu *et al.*, 2008; Limam, Sadok & El Abed, 2008). Enzymatic proteolysis is an attractive means of producing protein concentrates with interesting nutritional properties from by-products. Most of the time, hydrolysis of marine by-products are conducted by using, large spectra proteases that work in neutral or alkaline environment as their effectiveness of protein recovery and peptides generation were largely reported. However, in the case of hard tissues like the exoskeleton of crustacean such processing conditions may be not sufficient to recover additional valuable component as the chitin for example.

The objective of this study was to check the effectiveness of proteases that work at extreme pH (over 9 or below 4) on matter solubilization and distribution and to produce valuable fractions that could be further upgraded. Long term proteolysis (22 hours) was thus conducted with 4 proteases, 2 acidic ones (pepsin and Novozym 37020) and 2 alkaline ones (Protex 6L and Delvolase).

2. Material and methods

2.1. Materials

2.1.1 Raw material

Heads of frozen aquaculture shrimp (*Penaeus monodon*, Family: *Panaeidae*, Order: *Decapoda*, Class: *Malacostraca*, Phylum: *Arthropoda*) were collected from a processing factory (UNIMA®, Madagascar) on August 2005. They were packed in plastic bags and stored at . 20 °C until use. Maximal time of storage was 3 months.

2.1.2. External enzymes

All the enzymes used here are endopeptidase ones.

Pepsin from porcine stomach mucosa (EC 3.4.23.1) was purchased from Sigma Aldrich (Steinheim, Germany).

Novozym 37020 is a large spectra protease produced by fermentation, it was provided by Novozymes (Bagsvaerd, Denmark).

Protex 6L (EC 3.4.21.62) is an alkaline serine endopeptidase derived from a selected strain of *B. licheniformis*, it was provided by Genecor International (New York, USA).

Delvolase (E.C 3.4.21.14) is an industrial enzyme mixture from DSM Food Specialties (Seclin, France); the main constituent is a serine protease made from *Bacillus licheniformis*.

2.2. Hydrolysis

Figure 1 outlines the overall process employed in the production of shrimp head hydrolysates. The frozen and minced shrimp heads were stored overnight at 4°C for thawing and homogenized with MilliQ-purified water (ratio 1:1, w/v). For each hydrolysis, 100 g sample head were used. The mixture was directly poured into a 300 mL closed glass vessel with a double jacket to enable thermal exchanges (heating or cooling). In order to ensure a good proteolysis and despite the lack of data on this substrate, hydrolysis reactions were performed at pH and temperature known to be optimal for enzyme activity on reference molecules: Pepsin: pH 2, 40 °C; Novozym 37020: pH 3, 50 °C; Protex 6L: pH 9.5, 60 °C; Delvolase: pH 10, 60 °C. Before hydrolysis, mixing was performed for an initial 15 min to adjust the pH (by adding 1 M HCl or 2 M NaOH) and to obtain the desired temperature (using a water bath). Hydrolysis was initiated by adding enzymes. Due to the difference of specific activity of enzymes, different ratio enzyme/substrat were used to counterbalance those variations. Thus, 5 g/kg of Pepsin or Novozym 37020; 10 g/kg of Protex 6L and 20 g/kg of Delvolase were adopted. The mixtures were continuously stirred at 300 rpm for 24 hours. Temperature and pH were regulated (by using 2 M HCl or 2 M NaOH).

The degree of hydrolysis (DH) is defined as the % of peptide bonds cleaved during the enzymatic reaction. There is proportionality between the number of peptide bonds cleaved and the acid or base consumption which is given by the following equation:

$$\%DH = h/h_{tot} \times 100$$

where, h, number of peptide bonds broken; $h_{tot} = 8.6$ meq/g, total number of peptide bonds in substrate (Adler-Nissen, 1986).

According to Adler-Nissen (1977), when the reaction pH is above the pK_a of the α -NH group, then the equation is:

$$\%DH = h/h_{tot} \times 100 = (BN_b/\alpha h_{tot}MP) \times 100$$

where B = X ml, base consumption in ml; $N_b = 2$ N NaOH, normality of the base; α is the average degree of dissociation of the α -NH groups (see below); MP is the mass of protein in grams ($N \times 6.25$) (Adler-Nissen, 1977).

According to Zhao *et al.* (1996), when the reaction pH is below the pK_a of the α -NH group, the DH value can be estimated from the equation

$$\%DH = h/h_{tot} \times 100 = (AN_a / (1-\alpha)h_{tot}MP) \times 100$$

where A is the acid consumption in ml, N_a is the normality of the acid, α and MP are similar as above (Zhao, Sannier & Piot, 1996).

The degree of dissociation was found in the following way

$$\alpha = (10^{pH-pK}) / (1 + 10^{pH-pK})$$

The pK values at different temperatures T (Kelvin) were calculated from Steinhard and Beychok (1964):

$$pK = 7.8 + \frac{(298 - T)}{298T} \times 2400$$

After 22 hours (1335 min), hydrolysis were stopped by enzyme inactivation: addition of 5 M NaOH to reach neutrality for Pepsin; heating at 85 °C for 25 min for Novozym 37020, 85 °C for 20 min for Protex 6L and 90 °C for 20 min for Delvolase. After cooling at room temperature, the hydrolysates were centrifuged at 10.000 g for 20 min. Two fractions were thus collected: the insoluble sludge and the soluble aqueous phase. Each fraction was then freeze-dried and stored at . 20 °C until further analyses.

2.3. Chemical analysis

All the analyses were performed in triplicate except the amino acid profile and the molecular weight distribution.

Moisture content was determined by oven drying samples at 105 °C until constant weight (Association of Official Analytical Chemists [AOAC], 2005). Ash content was carried out by ignition of samples at 600 °C for 24 h (AOAC, 2005). The total nitrogen content of the non-hydrolyzed raw material, aqueous phase and insoluble sludge generated by hydrolysis was determined using the Kjeldahl method (Crooke & Simpson, 1971). Crude protein was estimated by multiplying the total nitrogen content (%N) by the factor 6.25.

Amino acid composition was determined for the raw (non. hydrolysed) material and hydrolysates according to a previous study (Kechaou *et al.*, 2009). Briefly, ten milligrams of freeze-dried samples were previously digested with HCl under nitrogen atmosphere for 18 hours. Then, the samples were completely dried under a nitrogen atmosphere and subsequently diluted by adding 2.5 ml water. The amino acid analysis was thus performed using the EZ:faast[®] procedure (Penomenex, USA) consisting of a solid phase extraction

step followed by derivatization and liquid/liquid extraction. An aliquot of the organic phase was analysed on a GC-FID system (Perkin Elmer Autosystem XL). The injector temperature was maintained at 250 °C while detection occurred at 320 °C. Separation was achieved using a Zebro ZB-AAA GC column (10 m x 0.25mm) with the oven temperature linearly increasing from 110 to 320 °C at 32°C/min. Helium was employed as the carrier gas at a constant flow rate of 1.5 mL/min. The amino acids were quantified by their response factor relative to the internal standard norvaline added at a concentration of 200 µmol/L.

Lipids were extracted according to the Folch procedure (Folch, Lees & Stanley, 1957). Total lipid content was determined gravimetrically after solvent extraction and evaporation.

2.4. Determination of Molecular Weight

The molecular weight distribution of peptides in the hydrolysates was analysed by gel filtration chromatography. The molecular weight fractions were separated using a high performance liquid chromatography (HPLC) system equipped with a size exclusion column (Superdex peptide 10/300 GL). The mobile phase consisted of water with trifluoroacetic 1 g/L and acetonitrile 5 g/L (70:30), the flow rate was 0.5 mL/min. The chromatography was monitored by measuring the absorbance at 214 nm. The column was calibrated with standards: Ribonuclease A (13700 Da), Aprotinin (6500 Da), Renin (1760 Da), Vasopressine (1084 Da) and Leucine (294 Da). The molecular weight ranges of the different fractions were based on the retention times of the collected fractions and determined from a standard curve.

2.5. Statistical analysis

All data presented are means \pm standard deviations. Analysis of variance (One way ANOVA) were performed by Statgraphics Plus version 5.1 (Manugistics Inc., USA). The method used to discriminate among means was Fisher's least significant difference (LSD) procedure. Mean were accepted as significantly different at 95% level ($p < 0.05$).

3. Results and discussion

Without any antimicrobial treatment (of the raw material and during the hydrolysis) bacterial proliferation may have occurred. Microbial compounds that can be microbially formed during the process may affect the global odour of the products (due to complex mixtures of volatile compounds such as unsaturated carbonyl compounds and alcohols with 6, 8 or 9 carbon atoms). However, non significant modification of the overall aroma was percept even after 22 hours of hydrolysis confirming a reduced bacterial proliferation under such conditions of hydrolysis.

3.1. Proximal composition of shrimp head

On dry weight basis, proteins were found to be the major constituents of shrimp head ($49.1 \pm 0.9\%$) while lipids and ashes were equivalent and represented around 17-19% ($16.6 \pm 0.2\%$ and $18.7 \pm 0.1\%$ respectively). Chitin was considered as the residue and thus estimated to be around 16%.

3.2. Degree of hydrolysis

During hydrolysis, shrimp head turned into a free flowing liquid. Hydrolytic curves corresponding to Pepsin, Novozym 37020, Protex 6L and Delvolase are reported in Figure 2. Whatever the enzyme used, first order kinetics was observed with an initial rapid phase where a large number of peptides bonds are broken (rapid consumption of HCl or NaOH), followed by a decreased rate of hydrolysis and finally approaching a stationary phase where no apparent hydrolysis takes place. Such hydrolysis curves are similar to those previously published on herring muscle and head (Liceaga-Gesualdo & Li-Chan, 1999; Sathivel *et al.*, 2003), salmon head (Gbogouri, Linder, Fanni & Parmentier, 2004; Sathivel, Smiley, Prinyawiwatkul & Bechtel, 2005) and cuttlefish and sardine viscera (Dumay, Donnay-Moreno, Barnathan, Bergé & Jaouen, 2006; Kechaou *et al.*, 2009) for example. At higher %DH, product inhibition may occur due to the high concentration of soluble peptides that can act as effective substrate competitors (Weil, 2005).

After 22 hours of hydrolysis, %DH varied from 18.6%DH (Protex 6L) to 36.5%DH (Pepsin). Such results are in accordance with previous proteolysis conducted on marine substrates like shrimp discards (%DH = 30% with Alcalase; Synowiecki & Al-Khateeb, 2000) or sardine heads (%DH = 20% with Protamex; Dumay *et al.*, 2006).

Regarding the kinetics, in our conditions, Pepsin was found to be the most efficient in hydrolysing shrimp head leading to the highest %DH but also reaching 33.5%DH in less than 3 hours (167 min). Thus, among the four enzymes tested here, Pepsin was the most performing enzyme in term of proteolysis (peptidic bond broken) and reaction rate (time of proteolysis). Despite a lowest maximal %DH (22.3%DH) at the end, Novozym 37020 appeared to be second most efficient enzyme as, after 3 hours, 17.9%DH was observed (92% of the final value). Both of these enzymes are acidic ones which could explain their highest proteolytic activities on shrimp heads. It is well known that protein solubility and conformation is influenced by pH condition: each protein has an isoelectric pH where their insolubility is maximal. As far as the pH condition is different of isoelectric pH of the proteins, their solubility is increased, improving their capacity to interact with the enzyme. Some proteins, like glutenin for example, have a maximal solubility at pH 2 or pH 12. Our results suggest that shrimp head proteins are glutenin-like as far as isoelectric pH is concerned.

For the alkaline proteases Protex 6L and Delvolase, lowest efficiencies (18.6 and 23.7 %DH) and reaction rates (73.5 and 56.2% of the final value after 3 hours) were obtained. Moreover, even after 22 hours proteolysis seemed to continue as little increases of %DH were observed.

3.3. Dry matter distribution

Without hydrolysis (i.e. in the crude head shrimp), after the centrifugation step, only 31.6% of dry matter was located into the soluble phase while 68.4% remained insoluble (Figure 3). After conducting hydrolysis on shrimp head samples, the dry matter distribution was mainly located in the supernatant, regardless to the enzyme used. This illustrates the solubilization of the matter under the action of the proteases as previously noticed notably on marine by-products (Aspmo, Horn & Eusink, 2005; Dumay *et al.*, 2006; Kechaou *et al.*, 2009). However, slight differences were observed according to the enzyme. Indeed, Protex 6L and Pepsin were found to be the most efficient in terms of dry matter solubilization with 64.1 and 63.5% of dry matter located in supernatant phase after hydrolysis while Novozym 37020 and Delvolase have completed only 53.5% and 51.6% of solubilization respectively. However, it is important to notice that despite the long time of hydrolysis (22 hours), at least 36% of shrimp head remained insoluble due to the presence of exoskeleton which are not easily hydrolysed. However, as previously noticed, such insoluble phases are of interest due to

their content in non modified chitin that can be easily recovered from sludge by classical chemical methods (Gildberg & Stenberg, 1991; Gildberg & Stenberg, 2001).

In our conditions, slight increases of salts (from 3 to 6.2%) were observed due to pH regulation of proteolysis by NaOH or HCl

3.4. Proximate composition

The average chemical composition (based on triplicates) of the freeze-dried soluble and insoluble fractions after 22 hours of hydrolysis are presented on Table 1. Regardless to the enzyme used, according to statistical analysis, supernatants were found to be rich in protein (48.1 to 64.7%) and poor in lipid (1.2 to 3.8%). As expected, those results illustrate the effectiveness of proteases to solubilize proteins but such enzymes seem here ineffective to recover lipids of shrimp heads into the resulting soluble phases.

Such high levels of protein recovery into soluble phase have been previously observed either for hydrolysates of shrimp discards (Synowiecki & Al-Khateeb, 2000), for shrimp head silage (Oliveira Cavalheiro *et al.*, 2007) and after fermentation of shrimp biowaste (Bueno-Solano *et al.*, 2009). However, in some cases, higher protein recovery rates have been obtained (69 to 91% on dry weight basis) (Gildberg & Stenberg, 2001; Ruttanapornvareesakul *et al.*, 2005; Limam *et al.*, 2008) which could be due to biochemical differences (proximate composition of raw material) and processing conditions (enzyme type, temperature, pH) as previously noticed (Slyzite, Dauksas, Falch, Storro & Rustad, 2005, Dumay *et al.*, 2006).

The few amounts of lipid into the supernatants are in accordance with previous studies on shrimp waste hydrolysates where less than 1% of lipids were quantified (Gildberg & Stenberg, 2001; Ruttanapornvareesakul *et al.*, 2006). Regardless to the enzyme used, the major part of lipids remained into the sludge (95.7, 88.9, 78.1 and 84.5% for Pepsin, Novozym 37020, Protex 6L and Delvolase respectively).

Regarding the ash content of the supernatant, it ranged from 24% (Delvolase) to 41% (Pepsin). According to enzyme used, ash contents are significantly different ($P < 0.05$). Anyway, hydrolysis under acidic pH led to hydrolysates richer in minerals (33% and 41% respectively for Novozym 37020 and Pepsin) while proteolysis under alkaline conditions led to a lower level of minerals (24% and 29% respectively for Protex 6L and Delvolase). Such results are expected as it is well known that demineralisation of shrimp carapace occurs at low pH and this is the basis of the chitin extraction by chemical treatments (Cauchie, Jaspars-Versali, Hoffmann & Thomé, 2002; Paulino, Simionato, Garcia & Nozaki, 2006; Pillai, Paul & Sharma, 2009; Sagheer, Al-Sughayer, Muslim & Elsabee, 2009; Yen, Yang & Mau, 2009) or by fermentation process (Bhaskar *et al.*, 2007; Cira *et al.*, 2002; Jung, Kuk, Kim & Park, 2005; Pacheco *et al.*, 2009). In addition, low pH increases the solubility of insoluble salt of Calcium (Jung & Kim, 2007). It has to be noticed that, as expected, addition of acid or alkaline during hydrolysis increases ash content in hydrolysates (Thiansilakul, Benjakul & Shahidi, 2007). Indeed, regarding to the DH which is related to the quantity of acid or alkaline added, Pepsin which induced to the highest DH generated the hydrolysate richest in ash. Inversely, hydrolysis with Protex 6L produced the hydrolysate poorest in ash, while the DH obtained was the lowest.

Such long time hydrolysis have led to a protein enrichment of the supernatants (Pepsin except) and a great reduction of their fat contents. Indeed, by comparison to non-hydrolysed material, the lipid amount into supernatant was reduced by up to 16 with Pepsin (16.6 g of lipid per 100 g of dry head and 1.4 g of lipid per 100 g of supernatant). This factor reduction is around 5 for the other enzymes (Novozym 37020, Protex 6L and Delvolase). However, such proteolysis and notably the one under acidic conditions have led to a mineral

enrichment of the soluble phases. Consequently, the resulting sludges were found to be poorer in proteins and richer in lipids by comparison to raw material. This confirms that such proteolysis can lead to very different fractions on a biochemical point of view.

3.5. Nitrogen recovery

Nitrogen recovery indicates the percentage of nitrogen solubilized into hydrolysate to total nitrogen in the raw material (Figure 4). After 22 h of hydrolysis, the amounts of soluble nitrogen were 79.5, 73.9, 70.3% and 54.9 with Protex 6L, Pepsin, Delvolase and Novozym 37020 respectively.

Such levels of nitrogen recovery are in accordance with those previously observed in similar studies on shrimp head (Gildberg & Steinberg 2001; Ruttanapornvareesakul *et al.*, 2005; Limam *et al.*, 2008) but also on other kind of marine material like fish (Kristinsson & Rasco, 2000, Jung & Kim, 2007), cuttlefish and sardine (Kechaou *et al.*, 2009). This illustrates how proteases can be efficient for recovering soluble nitrogen by breaking peptidic bonds but at various rates.

By comparison to the observed %DH (see above) some discrepancies appear here due to the fact that %DH do not reflect the protein solubilization but give the proportion of peptidic bonds cleaved under protease actions. Thus, if Pepsin was found to be an efficient enzyme for cleaving bonds or recovering the proteins this was not the case for Protex 6L which was found to be the best for cleaving but the worst for recovering nitrogen. This illustrates the complementarities between those 2 indicators and the capacities of the proteases to cleave or not peptidic bonds of already solubilized proteins leading to the reduction of the molecular size distribution of the soluble peptides. As all those enzymes are endoproteases, cleaving peptidic bond will in some case be not sufficient to induce a higher nitrogen recovery as the resulting peptides are not small enough to become soluble after centrifugation.

Some authors have observed that, during shrimp biowaste fermentation, protein solubilization was enhanced at higher pH (Bhaskar *et al.*, 2007; Rao *et al.*, 2000). This is partially confirm here where the alkaline proteases (Protex 6L and Delvolase) were found efficient while Novozym 37020 not. However, Pepsin which has worked at pH=2 counterbalance this. Further studies are thus needed to confirm or not such pH influence.

3.6. Molecular weight

Shrimp head hydrolysates were separated using gel chromatography to compare their peptide size composition. The resulting chromatogram of Pepsin hydrolysate is presented on Figure 5 as an example. The data treatment of those chromatograms (area under the curve) is presented on Figure 6.

Enzymatic hydrolysis of shrimp head has enhanced the solubility of proteins but has also cut them into shorter peptides. Indeed, 82.5 to 92.7% of soluble peptides had a molecular weight below 1000 Da and 38.1 to 49.6% had their molecular weights ranging from 250 to 500 Da. However, regarding to the enzyme used, slight differences can be noticed. Indeed, after 22 hours of proteolysis with Delvolase, less than 2% of the soluble peptides were found bigger than 2000 Da while 3 times more were quantified into Pepsin supernatant. (7.5%). With Protex 6L, up to 17% of peptides under 250 Da were detected while they represented less than 10% in Delvolase soluble phase. Those results are similar to some previous studies with shrimp head hydrolysate where the major peptides were found between 1400 to 300 Da (Ruttanapornvareesakul *et al.*, 2006).

It is well known that the smaller the peptides are, the highest their absorption by the intestinal tract will be (He *et al.*, 2006; Plascencia-Jatomea, Olvera-Novoa, Arredondo-Figueroa, Hal & Shirai, 2002). In addition, numerous short chain peptides have been identified, notably into marine hydrolysates, for their biological properties (Chabeaud *et al.*, 2009; He *et al.*, 2006; Je, Park, Kwon & Kim, 2004; Je, Qian, Byun & Kim, 2007; Ravallec-Plé & Van Wormhoudt, 2003). Thus, such enzymatic hydrolysis can be a good way of upgrading shrimp heads by producing hydrolysates rich in small peptides for nutritional and biological purposes.

3.7. Amino acid composition

The total amino-acid compositions of raw material and the 4 hydrolysates after 22 hour-hydrolysis are presented in Table 2.

As expected, the proteolysis has led to an amino acid enrichment in the supernatant by comparison to the soluble phase recovered after centrifugation of the raw material. Indeed, starting with 177 mg of amino acid per gram of dry weight, up to 398 mg were obtained into the Protex 6L supernatant. However, this enrichment was not similar whatever the enzymes, as only 239 mg were quantified into the Novozym 37020 supernatant, 271 and 303 mg into the Pepsin and Delvolase ones (and 398 mg into the Protex 6L one). Those results fit with nitrogen recovery rates as the nitrogen is mostly under the peptidic form.

From 14 to 15 different amino acids were detected into those different fractions as in some cases, some amino acids were not anymore detected into the soluble phases (Methionine with Novozym 37020 and Histidine with Protex 6L). It is reported that alkaline condition at pH under 9.5 may lead to the desamination of basic amino acids. Histidine destruction after hydrolysis with Protex 6L may result from that. However, with or without proteolysis, and whatever the enzyme, the proportion of essential amino acids (EAA) was found to be stable, between 35 to 40%. 8 essential amino acids were identified in raw material and shrimp heads hydrolysates. Tryptophane was not identified as well as cysteine which is conditionally essential for the reason that it is needed at high amounts in some physiopathological circumstance. The absence of tryptophan in shrimp heads and shrimp heads hydrolysates is explained by its destruction at acidic condition during amino acid analysis. Cystein is sensitive to oxidation and under thermal treatment at alkaline condition, converted to dehydroalanine or lanthionine (Cheftel, Cuq & Lorient, 1985). Those reactions may be responsible of the destruction of cysteine in shrimp heads and shrimp heads hydrolysates.

In terms of concentrations, in raw material and hydrolysates, more than 60% of the total amino acids were constituted by Glutamic acid (14.17%), Aspartic acid (10-15%), Glycine (8-12%), Alanine (7-12%), Leucine (8-9%) and Proline (7-10%). Such results are in accordance with previous results on shrimp biowaste hydrolysates (Armenta & Guerrero-Legarreta, 2009; Ruttanapornvareesakul *et al.*, 2006; Synowiecki & Al-Khateeb, 2000) where Aspartic acid and Glutamic acid were detected in high amounts. However, some discrepancies can be noticed regarding the enzyme. Indeed, the alkaline enzymes (Protex 6L and Delvolase) were found to be the most efficient for the recovery of Methionine (increase factor of 7 and 5 respectively) and on Serine (increase factor of 4 and 3 respectively). At the opposite, Novozym 37020 seems to be non appropriate for the extraction of Methionine (not anymore detected) and Tyrosine, Alanine and Phenylalanine with lower values compared to raw material (this is also the case for Protex 6L with Histidine).

Among the major amino acids detected into the 4 hydrolysates, some have an important nutritional interest. Indeed, Leucine is an essential one that has to be given into feed and foodstuffs while Glutamic acid, Aspartic acid, Alanine and Glycine are known to be flavour enhancers (Heu *et al.*, 2003; Jung Nim, T Watanabe, Endoh, K Watanabe & Abe, 2002).

Moreover, the levels of EAA are in accordance with the nutritional recommendation of the FAO (Cao, Zhang, Hong & Ji, 2008). At last, the increase of the Lysine content is of interest notably for feed purpose as this amino acid is the limiting one in cereals and has to be added notably in poultry feed.

These results indicate that shrimp head hydrolysates can be a good source of high nutritional quality products for feed or food purposes.

4. Conclusion

Proteolysis is well known to be a gentle method for recovering proteins from marine by-products, and thus generate peptides with functional and biological activity, but also improving amino acid composition increasing that way the protein quality. Extraction of elements from hard tissues requires extreme environmental conditions. In this study, we have tried to gather the 2 hypothesis by conducting long time proteolysis with acidic or alkaline enzymes.

This study like previous ones confirms thus that enzymatic hydrolysis is an effective process to separate components from marine biomass for further upgrading. Whatever the protease used here, a great part of proteins was recovered in the aqueous phase as small size peptides with interesting level of essential amino acids. Variations were however observed regarding the enzymes used and notably the alkaline or the acidic ones. One of the main differences was on the mineral content of the resulting phases which validates that acidic proteases can help to recover calcium into aqueous solutions and thus contribute to increase the nutritional interest of such hydrolysates.

This study has confirmed that enzymatic hydrolysis could be a valuable upgrading process for shrimp wastes notably the head as it is quite simple to implement and because it generates rich protein hydrolysates with low fat content that can be useful for food and feed purpose. Additionally the insoluble fractions contain valuable component such as lipids, insoluble proteins and partially demineralised and deproteinised chitin that can be further separated.

Acknowledgements

The authors wish to thank AUF (Agence Universitaire pour la Francophonie) for their financial support.

This study is part of the actions conducted by the members of the SEA^{PRO} network (www.seapro.fr) which aims to promote a better use of the marine biomass with sustainable process.

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Tables

Table 1: Chemical composition of fractions of hydrolysed shrimp head with commercial enzyme, g/100g on dry basis.

Tabla 1: Composición química de las fracciones de cabezas de gamba hidrolizadas con enzima comercial, g/100g en base seca.

Fractions	Enzyme	Protein (%)	Lipid (%)	Ash (%)	Chitin (%)
Soluble	Pepsin	48.12 ± 1.57	1.17 ± 0.34	41.1 ± 0.46	9.61
	Novozym 37020	51.59 ± 0.01	3.01 ± 2.28	32.93 ± 0.06	12.47
	Protex 6L	63.86 ± 0.21	3.81 ± 0.21	23.86 ± 0.27	8.47
	Delvolase	64.73 ± 0.83	3.57 ± 0.02	28.62 ± 0.93	3.08
Insoluble	Pepsin	29.57 ± 0.29	36.20 ± 0.32	20.31 ± 0.12	13.92
	Novozym 37020	47.14 ± 0.32	29.19 ± 1.02	9.24 ± 0.62	14.44
	Protex 6L	29.44 ± 0.27	24.31 ± 1.11	36.80 ± 0.56	9.45
	Delvolase	29.19 ± 0.96	20.82 ± 0.94	30.79 ± 0.16	14.20

Table 2: Amino acid composition in raw material and hydrolysates, mg/g on dry weight basis.

Tabla 2: Composición en aminoácidos para la materia prima y los hidrolizados, mg/g en base seca.

Amino acid	Raw material	Pepsin	Novozym 37020	Protex 6L	Delvolase
Leucine	15.72	22.27	19.65	35.37	24.89
Phenylalanine	11.55	18.15	3.30	24.75	18.15
Valine	9.36	12.87	16.38	24.57	19.89
Lysine	8.76	17.52	16.06	16.06	11.68
Isoleucine	7.86	14.41	13.10	23.58	18.34
Threonine	5.95	10.71	15.47	20.23	14.28
Histidine	1.55	4.65	6.20	0.00	6.20
Methionine	1.49	4.47	0.00	10.43	7.45
Essential amino acids (EAA)	62.24	105.05	90.16	154.99	120.88
Glutamic acid	24.99	39.69	39.69	60.27	49.98
Alanine	21.36	26.70	16.91	32.04	24.03
Aspartic acid	21.28	41.23	23.94	43.89	35.91
Glycine	18.75	21.00	28.50	35.25	27.75
Proline	17.25	21.85	24.15	27.60	24.15
Tyrosine	7.24	10.86	5.43	18.10	7.24
Serine	4.20	4.20	9.45	16.80	13.65
Non essential amino acids (NEAA)	115.07	165.53	148.07	233.95	182.71
%EAA	35.10	38.82	37.85	39.85	39.82
EAA/NEAA	0.54	0.63	0.61	0.66	0.66

Figures

Figure 1: Hydrolysis process flow diagram.

Figura 1: Organigrama del proceso de hidrólisis.

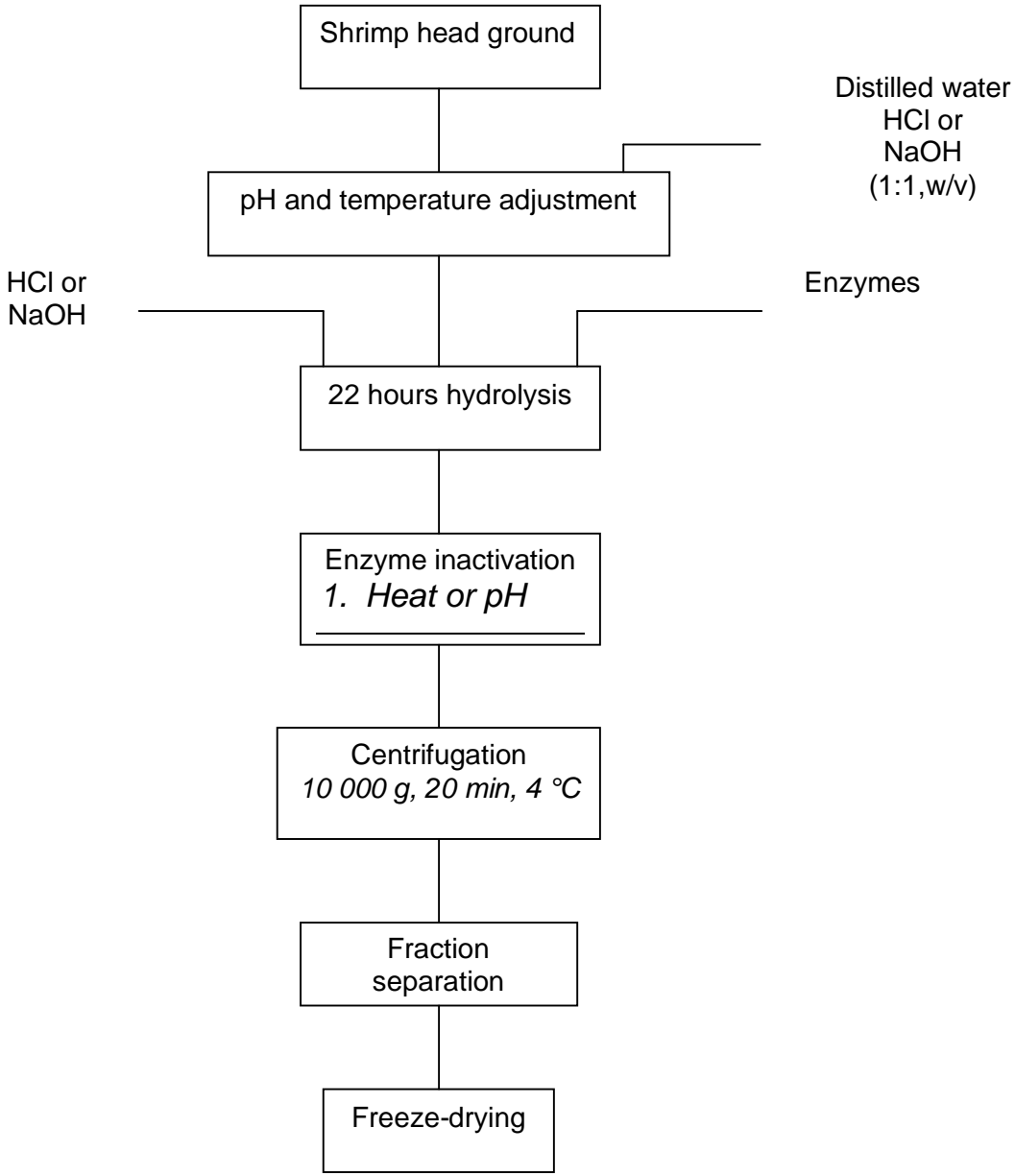


Figure 2: 22 hours hydrolysis curves of shrimp head with commercial enzymes.

Figura 2 : Curvas de la hidrólisis de 22 horas de cabezas de gamba con enzimas comerciales.

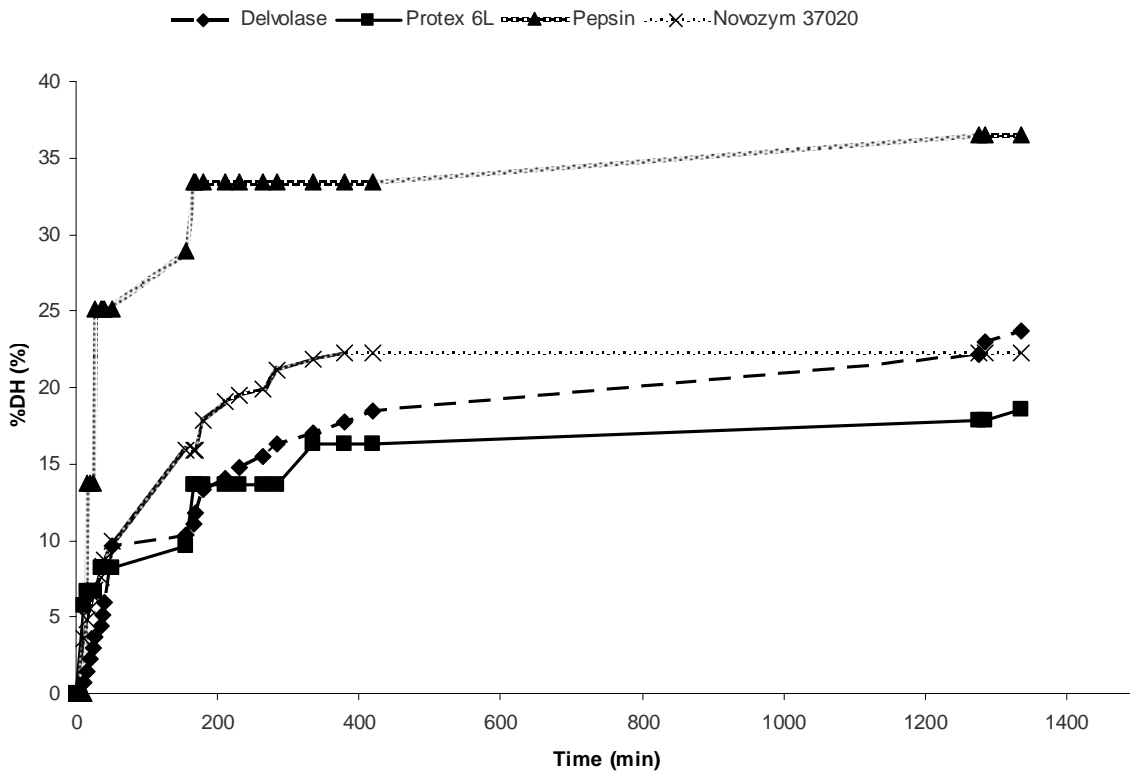


Figure 3: Dry matter distribution in supernatant and sludge after 22 hours of hydrolysis of shrimp head with commercial enzymes.

Figura 3: Distribución de la materia seca en el sobrenadante y residuo después de 22 horas de hidrólisis de cabezas de gamba con enzimas comerciales.

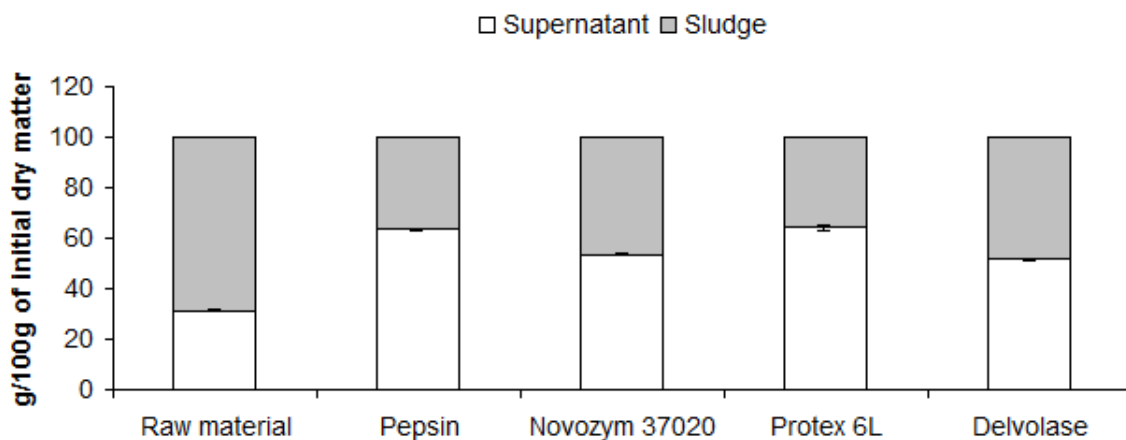


Figure 4: Nitrogen distribution in supernatant and sludge after 22 hours of hydrolysis of shrimp head with commercial enzymes.

Figura 4: Distribución de Nitrógeno en el sobrenadante y residuo después de 22 horas de hidrólisis de cabezas de gamba con enzimas comerciales.

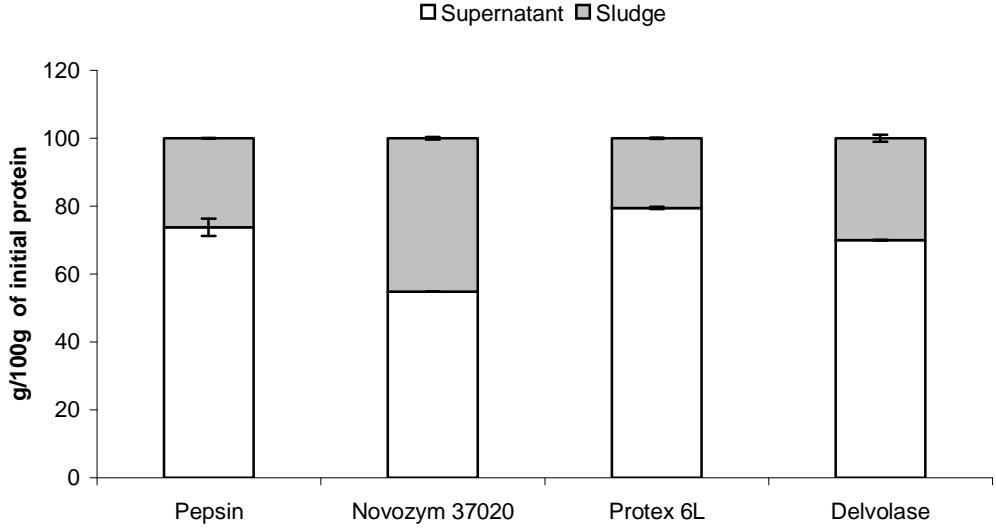


Figure 5: Chromatogram of shrimp head hydrolysate with Pepsin.

Figura 5: Cromatograma de hidrolizado de cabeza de gamba con Pepsina.

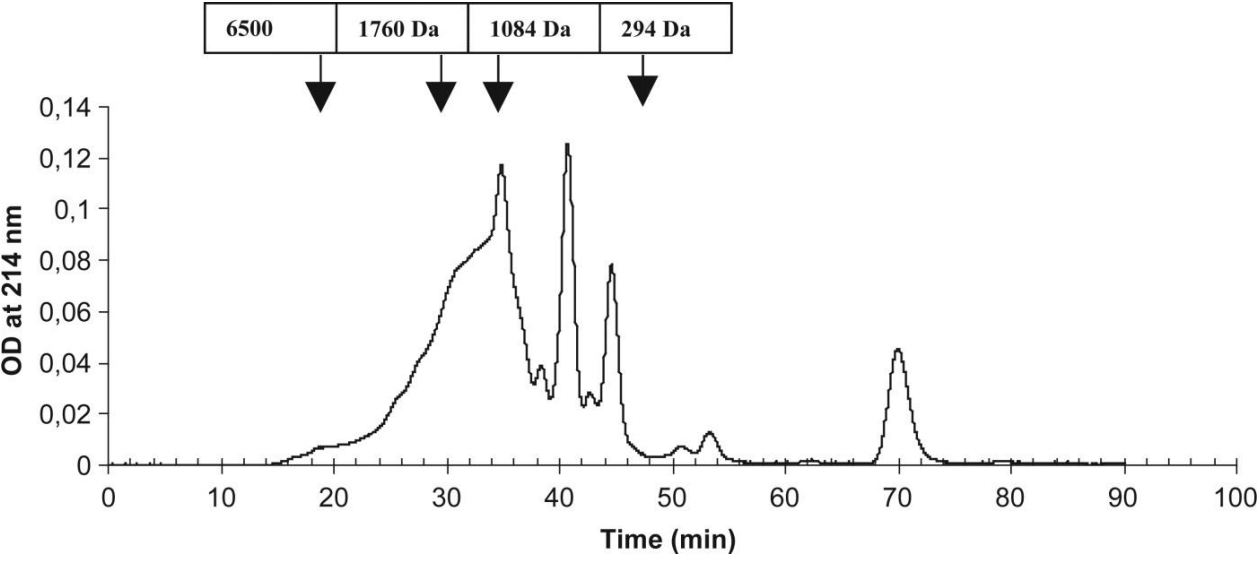
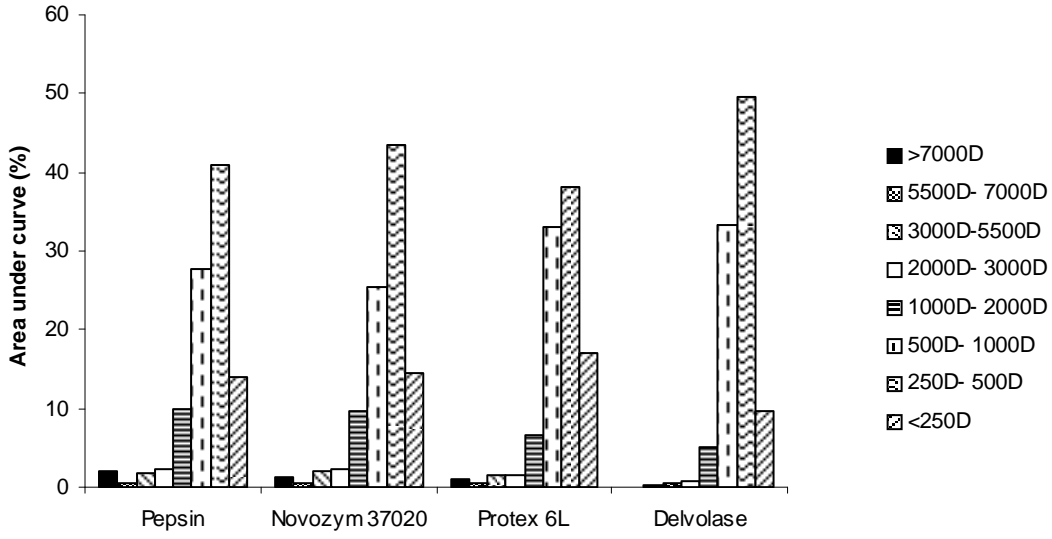


Figure 6: Peptides molecular weight distribution in hydrolysates.

Figura 6: Distribución de pesos moleculares en los hidrolizados.



Supplementary Table 1. Physico-chemical characteristics (ash and gluten content, gluten index) of meals, dough alveographic indices (W and P/L), and pasta value ($PV-K$) calculated according to the Equation (1).
 Tabla adicional 1. Características físico-químicas (ceniza y contenido de gluten, índice de gluten) de harinas, índices alveográficos de masa (W y P/L) y valores de pasta ($PV-K$) calculados según la ecuación (1).

Meal mixtures	Ash (g kg^{-1} dry weight)	Gluten (g kg^{-1} dry weight)	Gluten Index	b^*	W (J)	P/L (Pa mm^{-1})	$PV-K$
Semolina	8.3 ± 0.2a	100 ± 2d	80 ± 2a	21.6 ± 0.30h	146 ± 2f	2.02 ± 0.00a	34.8 ± 1.2a
5% TDWM-semolina	8.6 ± 0.2a	94 ± 2c	89 ± 3c	16.5 ± 0.22f	143 ± 2f	2.29 ± 0.10b	33.5 ± 1.0b
10% TDWM-semolina	9.0 ± 0.1b	93 ± 1c	87 ± 2b,c	14.8 ± 0.13f	124 ± 4d	3.59 ± 0.15c	28.3 ± 1.5c
15% TDWM-semolina	9.3 ± 0.2b	92 ± 3c	85 ± 2b,c	13.2 ± 0.12e	133 ± 1e	4.88 ± 0.20d	17.6 ± 1.7d
20% TDWM-semolina	9.6 ± 0.2b	92 ± 2c	85 ± 2b,c	12.5 ± 0.05d	114 ± 1c	5.71 ± 0.35e	6.3 ± 1.6e
30% TDWM-semolina	10.2 ± 0.2c	88 ± 1c	84 ± 1b	11.5 ± 0.06c	72 ± 2b	9.54 ± 0.10f	
40% TDWM-semolina	10.9 ± 0.3d	81 ± 2b	83 ± 2b	10.7 ± 0.20b	55 ± 3a	11.45 ± 0.10g	
50% TDWM-semolina	11.9 ± 0.5e	77 ± 0a	89 ± 3c	10.8 ± 0.20b	n.d.	n.d.	
TDWM	15.6 ± 0.3f	n.d.	97 ± 3d	9.8 ± 0.37a	n.d.	n.d.	

Note: n.d., not determinable.

Within each column, different letters indicate significant differences ($P < 0.05$).

Nota: n.d., no determinado.

En cada columna, letras diferentes indican diferencias significativas ($P < 0.05$).

Supplementary Table 2. Physico-chemical characteristics and optimal cooking time (OCT) of spaghetti made of semolina and TDWM.
 Tabla adicional 2. Características físico-químicas y tiempo óptimo de cocido (OCT) de espaguetis hechos con semolina y harina de trigo duro tostado.

Spaghetti samples	Moisture (g kg^{-1})	a_w	L^*	a^*	b^*	OCT (s)
Semolina	64.0±2.9a	0.51±0.01c	61.32±0.44e	2.3.±0.43b,c	36.93±0.31e	390±20a,b
5% TDWM-semolina	65.5±4.0a	0.47±0.00b	44.86±1.15d	4.47±0.22e	19.74±1.12d	390±20a,b
10% TDWM-semolina	63.7±1.6a	0.46±0.00a	36.50±1.83c	3.56±0.23d	9.72±0.83c	390±20a,b
15% TDWM-semolina	70.2±5.3a	0.47±0.02b	31.78±2.16b	2.41±0.11c	5.29±0.37b	420±10b
20% TDWM-semolina	61.0±8.9a	0.47±0.00b	30.89±0.70a,b	1.83±0.06b	4.90±0.09b	360±30a
30% TDWM-semolina	66.0±3.2a	0.47±0.02b	29.11±0.77a	1.07±0.06a	3.36±0.22a	390±20a,b

Note: Within the same column, different letters indicate significant differences ($P < 0.05$).

Nota: En cada columna, letras diferentes indican diferencias significativas ($P < 0.05$).