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Homologous ELISA procedure for the determination of penaeid shrimp vitellogenin

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Abstract

Vitellogenin (VTG) was isolated from the haemolymph of *Penaeus vannamei* by a three step procedure including ultracentrifugation, gel filtration and ion exchange chromatography. VTG was used to raise polyclonal antibodies that were purified by ion exchange chromatography. A two step competitive assay was developed in which VTG could be quantitated by its capacity to inhibit the binding of antibody to the VTG previously adsorbed onto a solid phase. Sensitivity from equilibrium and from non-equilibrium assays was 41 and 2.3 ng/ml respectively. Estimates of within-assay and between-assay variabilities of standard curves were 4.2 and 9.6% respectively. The inhibition curves for dilutions of haemolymph from vitellogenic females, egg yolk extracts and purified vitellin (VTL) were parallel to the standard VTG curve, haemolymph from immature females and males showed no cross-reactivity. The antibodies directed against VTG recognize but partially VTL as shown by displacement curves. Recovery tests were near 100%. Thus the procedure was considered to be suitable for the measurement of haemolymphatic VTG. The VTG enzyme-linked immunosorbent assay (ELISA) developed in this study was validated by detecting physiological VTG changes in female shrimps after being fed squid extracts.

Keywords: Penaeid, vitellogenin, vitellin, immunoassay (ELISA), antibody.

Un test ELISA homologue pour la détermination de la vitellogénine de crevettes pénéidés.

Résumé

La vitellogénine (VTG) de *Penaeus vannamei* a été purifiée à partir d'hémolymphe par une procédure en trois étapes comprenant l'ultracentrifugation, la filtration sur gel et la chromatographie d'échange d'ions. Un essai compétitif en deux étapes a été développé par lequel la VTG peut être quantifiée en fonction de sa capacité d'inhiber la liaison de l'anticorps à la VTG préalablement adsorbée à une phase solide. La sensibilité pour des essais en équilibre et non-équilibre a été respectivement de 41 et de 2,3 ng/ml. Les coefficients de variation intra-essai et inter-essais ont été respectivement de 4,2 et 9,6 %. Les courbes d'inhibition pour des dilutions de l'hémolymphe de femelles en cours de vitellogenèse, d'homogénats d'ovaire de ces dernières et de vitelline (VTL) purifiée ont été parallèles à la courbe étalon de VTG; en revanche, l'hémolymphe de femelles sexuellement immatures et de mâles n'ont pas montrée de réactivité croisée. Les anticorps dirigés contre la VTG ne reconnaissent que partiellement la VTL comme l'ont montré les courbes de déplacement. Les épreuves de surcharge ont été approximativement de 100 %. Ainsi la procédure a été considérée comme adéquate pour mesurer la VTG hémolymphatique. Le test enzymatique immuno-adsorbant (ELISA) développé dans cette étude a été validé physiologiquement en détectant des variations de VTG chez des crevettes femelles nourries avec des extraits de calmar.

Mots-clés : Pénéidé, vitellogénine, vitelline, immuno-essai (ELISA), anticorps.

INTRODUCTION

Vitellogenesis is a major metabolic event in the life cycle of female shrimp. It is characterized by vitellin (VTL) oocytic accumulation from the active uptake of a plasma precursor the vitellogenin (VTG) (Meusy, 1988) and/or intraovarian synthesis (Yano, 1988; Rankin *et al.*, 1989; Browdy *et al.*, 1990). This process is regulated by an inhibitory control exerted by the vitellogenic inhibiting hormone (VIH) which is synthesised and released from the organ-X sinus gland complex located in the eyestalk (Jugan and Soyez, 1985; Soyez *et al.*, 1987). Although it has become a regular practice to ablate one eyestalk from shrimp females to induce vitellogenesis, it has been reported that a number of disadvantages arise from this technique such as a lower fecundity and hatching rates as well as shorter periods of viable spawning (Lumare, 1979; Beard and Wickens, 1980, Nascimiento *et al.*, 1991). A more gradual way to induce vitellogenesis is required.

The utilization of squid as a regular component of the feed supplied to the reproductors to favour vitellogenesis has been extensively reported (AQUACOP, 1977; 1979, Chamberlain and Lawrence, 1981; Simon, 1982). However little research has been conducted to elucidate the nature of the stimulatory components.

We describe in the present study the development and validation of an homologous enzyme immunoassay for monitoring penaeid shrimp VTG in order to detect in a sensitive, specific and rapid way the physiological changes induced by the administration of squid extracts.

MATERIALS AND METHODS

Vitellogenin (VTG) and vitellin (VTL) purification

VTG was purified from haemolymph through 3 steps.

Isolation of the lipoprotein fraction by ultracentrifugation on a KBr density gradient

Haemolymph was adjusted to a density of 1.23 g/ml with a KBr solution ($p = 1.33$ g/ml) followed by a 24 h centrifugation at 120 000 g in a Beckman L5-656 ultracentrifuge equipped with a 50.2 Ti rotor.

Gel filtration

Lipoproteins were chromatographed on a 2.6 × 100 cm column of Sephacryl-300 HR (Pharmacia Fine Chemicals), bed vol. 500 ml. The elution was performed with a 0.1 M phosphate buffer pH 7.2 at a flow rate of 1.25 ml/min. The column eluent was monitored at 280 nm.

Ion exchange chromatography

Pooled fractions from the previous step were applied to a 2.6 × 8.3 cm column using DEAE-Spherodex (IBF biotechnology) as support. VTG was eluted by a linear salt gradient from 0 to 0.5 M KCl in a 50 mM Tris-HCl pH 8 buffer at a flow rate of 1 ml/min. Filtration through PD-10 (Sephadex G-25, Pharmacia) was carried out after steps 1 and 3 in order to desalt the fractions. Concentration of proteins after step 3 was performed by ultrafiltration using Immersible-CX ultrafilters (Millipore).

After two passages on ion exchange chromatography the VTG was identified as a major single symmetrical pic. This aspect was also confirmed by polyacrylamide gel electrophoresis (PAGE).

VTL was purified using the same procedure but ovaries were first rinsed in a physiological isoosmotic (950 mOsm/kg) saline and homogenized in a Potter-Elvejem in cold 50 mM phosphate buffer, phenylmethylsulfonyl fluoride (PMSF) 5 mM, pH 7.2. The homogenate was ultrasonicated and centrifuged at 4 000 g for 15 min to remove debris and fat material. The supernatant was purified like haemolymph.

Principle of the competitive VTG-ELISA

Purified VTG is first immobilized in a solid phase, then free VTG contained in samples or standards is added with a diluted antibody. The free VTG will compete with the coated VTG for the binding sites of the diluted antibody thus preventing a certain fraction of this antibody from being immobilized. The amount of antibody bound is measured in a subsequent step by an enzyme labelled second antibody. The enzyme activity detected is inversely related to the VTG concentration in the sample. The assay is calibrated using dilutions of standard VTG as competitor.

Stock solutions

Coating buffer

Sodium carbonate 0.05 M, buffer pH 9.6.

Blocking solution

Phosphate buffer saline (PBS) 0.01 M phosphate buffer pH 7.4, 0.15 M NaCl, 0.027 M KCl; Tween-20 0.05%; defatted dry milk 3.5%.

Washing solution

PBS, Tween-20 0.1%.

Assay buffer

PBS, Tween 0.05%, defatted milk 1.25%.

Conjugate buffer

PBS, Tween 0.05%, gelatin 5%.

Enzyme substrate solution

Orthophenylenediamine (OPD) 0.022 M, H_2O_2 0.035 M in phosphate/citrate buffer (Na_2HPO_4 $2H_2O$ 0.1 M, citric acid monohydrated 0.044 M) pH 5.5. This solution was prepared extemporaneously.

Substrate stopping solution

H_2SO_4 4 M, Na_2SO_3 5%.

General lines for the assay procedure

Immobilization of VTG

Nunc Maxisorp F96 microtiter plates were coated with 150 µl of reference VTG.

Saturation of unbound sites

Active sites left uncoated by the VTG were blocked by the addition of 200 µl of unreactive proteins (blocking solution).

Incubation of samples or reference VTG with anti-VTG antibody

In this step two modalities were assayed.

– Concurrent incubation, 75 µl of haemolymph samples (diluted at least 1 000 fold) thought to contain VTG or standard VTG were incubated with 75 µl of the specific antibody on the sensitized solid phase.

– Sequential incubation. Diluted haemolymph samples were preincubated with specific antibody in Eppendorf tubes followed by a differed transfer of 150 µl of the mixture to each coated well, then plates were incubated.

Addition of enzyme-labelled anti IgG

150 µl of goat anti-rabbit IgG peroxydase conjugate were added to each well and incubated.

Addition of enzyme substrates

Peroxydase activity was assayed by adding 150 µl of substrate solution to each well.

Arrest of the reaction

50 µl of substrate stopping solution were added to each well.

Optical density measurement

Plates were read on an Argus 300 automated microplate reader (Packard Instruments) linked to a microcomputer for data aquisition and treatment.

During incubations, plates were covered with parafilm, mixed on an orbital shaker and routinely incubated in a humidity chamber.

After the first four steps, plates were washed to remove unbound material. Washing was accomplished by turning the plates upside down and pouring out the contents, then the plates were flushed with the washing solution and finally, they were emptied further by striking them against paper towels and allowed to drain. The procedure was repeated 4 times each cycle.

The following parameters were estimated in each assay.

- BL: referring to the optical density (O.D.) from the wells devoided of coated VTG, indicating background reading.
- B: corresponding to the O.D. of samples or standards in competition with the coated VTG.
- BO: being the O.D. of wells without free VTG, thus providing maximum binding between coated antigen and specific antibody.

All the determinations for the calibration curve and unknowns were made in duplicate and the mean result was calculated. A number of quality criteria for validation of the assay were taken into account and experiments were so designed to optimize the conditions of every step.

Rocket immunoelectrophoresis (RIE)

Rocket immunoelectrophoresis was carried out by a modification of the original protocol of Laurell (1966). As a selected application to validate the assay we present partial results of an ongoing study for assessing the effectiveness of squid (*Loligo vulgaris*) extracts on the triggering of shrimp vitellogenesis.

Preparation of squid extracts

Initial extracts (lipids and a water-alcohol soluble fraction) were first obtained from a Bligh and Dyer extraction (1959) of whole squid. The rest of the extracts were obtained by further fractionation of the originals, recovering the nonsaponifible fraction of an alkalin hydrolysis in ethylacetate according to the eluotropic series of Makin (1974). The different polarity fractions were included at 1 ppt in a basal semipurified diet containing 44% protein, 8.6% lipids, 37.2% carbohydrates and 10.1% ash.

Expression of results and statistical analysis

VTG immunoassay data were analysed by unweighted least square linear regression after logit-log transformation or by cubic spline interpolation. The suitability of these transformations was tested by residual plot analysis and an F test for linearity after logit-log transformation. Slopes of standard curves were compared by the Student's *t*-test (Sachs, 1984) or an F test according to Dagnelie (1975).

The experimental design to study the effect of squid fractions was a randomized block design, the repetitions (2) along the time of each experimental series were considered as blocks. Comparison of the mean concentrations of different treatments was accomplished by ANOVA and Duncan's new multiple-range test (Steel and Torrie, 1980) and by a χ^2 test to assess the difference between proportions of individuals having responded to the fractions (Schwartz, 1984).

RESULTS

Optimization of the concentrations of reagents

A maximal absorbance BO value corresponding to 2 units of (O.D.) was selected in order to have some flexibility while optimizing the assay. Furthermore the reader gives a linear response up to this O.D.

As a first approach optimal concentrations of conjugate and specific antibody were estimated by a two dimensional titration (*fig.* 1). Assays indicated that antibody titers ranging from 1:50 000 to 1:70 000 yield a maximal absorbance of 2 O.D. for a conjugate dilution of 1:3 000. The latter titer was chosen for routine use. In a similar way VTG and specific antibody concentrations were simultaneously determined by chessboard titration (*fig.* 2). Plates were first coated with *log* dilutions to estimate the amount needed to reach the O.D. Once the range was identified, fine tuning was performed and a VTG amount of 75 ng/ml with an antibody dilution of 1:60 000 seemed to give good results, although other combinations of both reagents could have been chosen.

Parallelism

In order to determine the specificity of the assay for VTG, parallelism studies were conducted (*fig.* 3). Serial dilutions from purified VTL, egg yolk extracts and haemolymph from ripe females showed full parallelism with VTG standard calibration curve ($F=3.117$ N.S., $p>0.05$ with 3 and 17 d.f.) whereas specificity was further demonstrated by the absence of reaction with immature ovarian homogenates ($t=8.970$, 9 d.f.) and haemolymph ($t=10.010$, 9 d.f.) as well as with male haemolymph ($t=10.004$, 9 d.f.).

It is noteworthy that when precise doses of vitellin were incubated with coated VTG they generate an inhibition curve, that despite being parallel, showed only limited immunological potency in the assay (*fig.* 4).

Additionally the specificity of antibodies was corroborated by immunoenzymatic revelation after sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*fig.* 5).

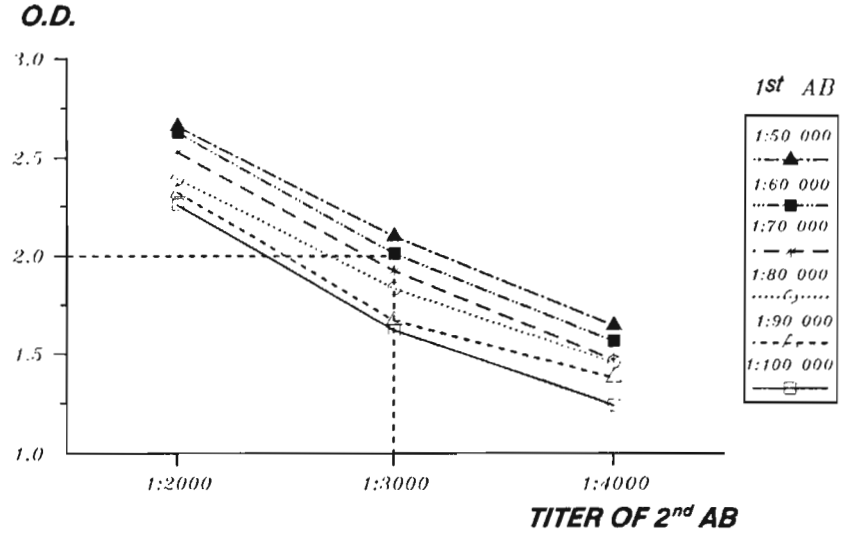

Figure 1. – Optimal concentrations of conjugate and specific antibody as determined by chessboard titration.

ELISA-RIE correlation

In order to test the reliability of the assay a direct comparison was made against RIE by measuring

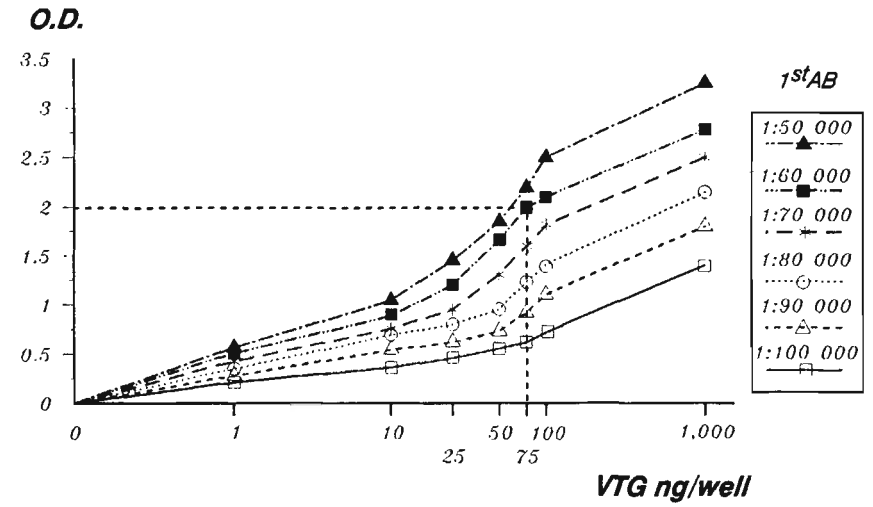

Figure 2. – Determination of the optimal concentration of vitellogenin (VTG) and first antibody. Wells were coated with serial dilutions of VTG and subsequently incubated, with serial dilutions of first antibody.

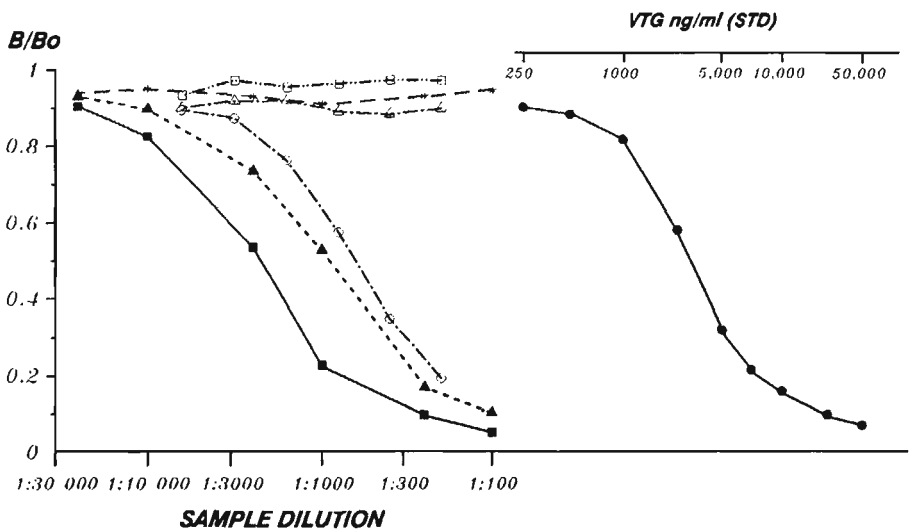

Figure 3. – Inhibition curves from serial dilutions of vitellin (VT) ■, eggyolk extract ▲, ovarian homogenate of immature females ∗, haemolymph from ripe females ○, immature females △, and males □, compared to reference vitellogenin (VTG) preparation ●. Each point is the average of duplicate determinations.

some standards and biological samples with both methods. The concentrations (ng/ml) as determined by ELISA were highly correlated with concentrations estimated by RIE ($r^2 = 0.976$, $n = 18$) the slope being very close to the unity (0.985) and an intercept of 498. This value was probably due to the difficulty of measuring rockets at the limit of the assay sensitivity.

Reproductibility

To determine the inherent variability within a single assay 10 samples (triplicates) containing known quantities of VTG were measured resulting in an average intra-assay coefficient of variation (C.V.) of 4.2%. The variability between assays was calculated by measuring the near 50% binding of standard curves in 10 separate assays. The C.V. estimated on this basis was 9.6%. Thus a sensitive and reproducible assay has been produced with a wide enough range for practical purposes.

Recovery

To determine the effect of variation in composition of the incubation media, plasma volumes (50 µl) of

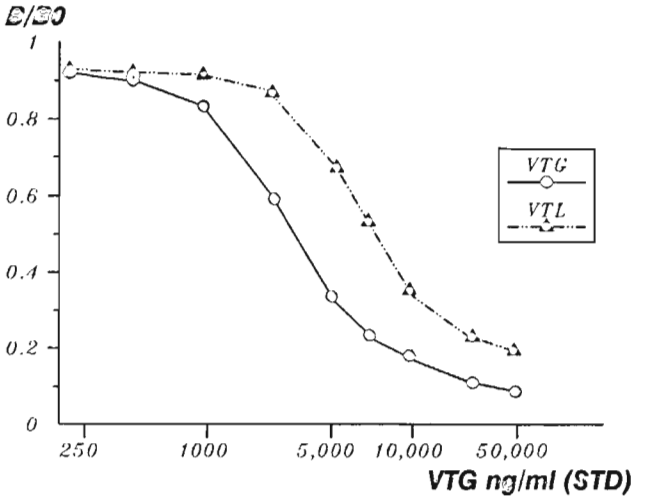

Figure 4. – Dose response inhibition curves for vitellogenin (VTG) and vitellin (VT).

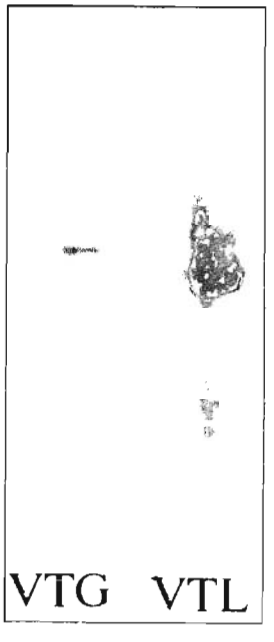

Figure 5. – Immunoblotting patterns of VTG and VTL revealed by IgG directed against purified vitellogenin. Revelation was accomplished with a goat-anti-rabbit IgG peroxydase conjugate, after SDS-PAGE of native molecules and a subsequent transfer to a nylon membrane by vacuum blotting.

juvenile females and males were spiked with increasing quantities of VTG (from 500 to 10 000 ng) which resulted in a calculated recovery of 94 and 102% respectively.

Working range

Routine calibration curve ranged from 250 to 50 000 ng, most sensitive and reliable part being between 1 000 and 10 000 ng/ml.

Statistical parameters

Mean, slope, intercept and determination coefficients calculated from regressions after linear and curvilinear transformations from 10 separate assays were respectively:

$$\text{Logit}(B/BO) = a + b\,\text{Ln}(\text{Dose}) \qquad (1)$$

$a = 8.6042 \pm 0.8594$
$b = -1.1262 \pm 0.1012$
$r^2 = 0.9839 \pm 0.0086$

$$B/BO = a\,\text{Ln}(\text{Dose}) + b\,\text{Ln}(\text{Dose})^2 + c\,\text{Ln}(\text{Dose})^3 + d \qquad (2)$$

$a = -0.1225 \pm 0.0676$
$b = 0.0021 \pm 0.0014$
$c = 0.00009 \pm 9.10^{-7}$
$d = 10.9007 \pm 0.9355$
$r^2 = 0.9907 \pm 0.0062.$

Sensitivity

Sensitivity defined as the smallest amount of VTG that can be distinguished from BO was calculated as twice the S.D. at zero dose. The sequential incubation modality greatly increased the sensitivity of the assay. Allowing antibody to equilibrate with soluble VTG before competition with coated VTG results in a shift of the calibration curve to the left and a steeper curve

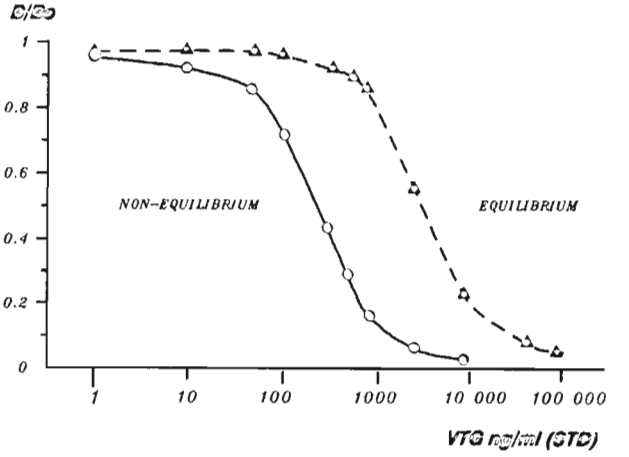

Figure 6. – Comparison of an equilibrium versus a non-equilibrium assay. Reference vitellogenin (VTG) was incubated concurrently with anti-VTG in coated VTG wells (equilibrium) or in separate tubes and subsequently added to VTG coated wells.

(*fig.* 6). Sensitivity for the equilibrium assay was 41 ng/ml whereas that of the non-equilibrium assay was only 2.3 ng/ml and the midpoint sensitivity of the latter (372.30 ng/ml) was a tenth of that of the former (3 773.65 ng/ml). By changing some of the conditions of the assay (adopting a non-equilibrium

system, using less coated VTG and with a less diluted conjugate) the sensitivity could be further enhanced up to 565 pg/ml.

Transformation

The legitimacy of the transformation for both models was tested over a combined calibration curve which integrates data of ten curves. Residual plot analysis showed scatter points with no systematic distribution on either side of the zero value, thus validating the transformation. Transformed data were linear as assessed by the F test: $F = 1\,233.79$ with 1 and 96 d.f.

Time courses and temperature kinetics

Time course trials and temperature kinetics were investigated in order to improve sensitivity and minimize assay time. Data from time course studies showed that VTG binding was completed after 4 h at 37°C or after 20 h for all temperatures tested (4, 25 and 37°C). Optimal saturation time for minimizing background was 1 h.

In the concurrent incubation assay, equilibrium was favoured by increasing incubation times and with high temperatures. We adopted a 2 h incubation period at 25°C to avoid high non specific binding.

Long preincubation times and short in-plate incubations at high temperatures increase sensitivity in such a way that small changes could be better defined. The former is because a short incubation of the mixture (VTG + anti-VTG) with the coated VTG allows binding of free antibody before VTG-antibody complexes are disrupted and a new equilibrium is established. The non-equilibrium assay results were similar to those reported in other studies (Farrington and Hymer 1987, Grimaud *et al.*, 1986, Signorella and Hymer, 1984; Salbert *et al.*, 1990).

The binding of the conjugate to the specific antibody reached the 2 O.D. value between 1 and 2 h at 37°C.

In other time course experiments, results indicated that it was necessary to let the reaction evolve for at least 30 min before stopping it and wait during 20 min before reading the plates. This allowed us to standardize the BO endpoint for the entire test.

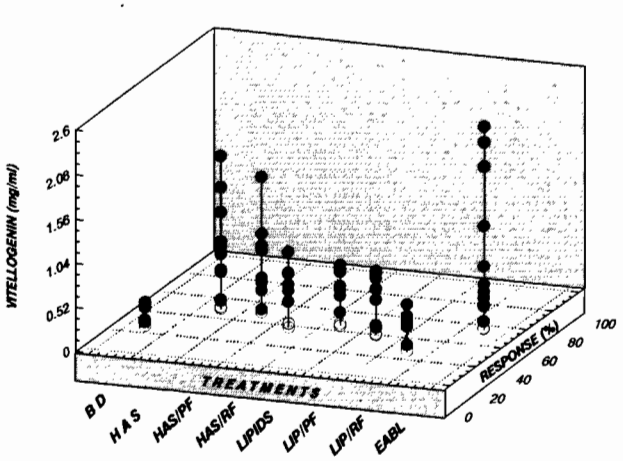

Figure 7. – Effect of squid extracts on shrimp vitellogenesis. Each mark ● represents an individual responding to the treatments, marks ○ are individuals showing only basal level and were not considered in the statistical analysis. Abbreviations: BD basal diet, HAS hydroalcohol soluble fraction, HAS/PF its polar fraction, HAS/RF residual fraction, LIP/PF and LIP/RF polar and residual fraction of lipid fraction, EABL eyestalk ablated females.

Applications of the competitive ELISA to monitor *P. vannamei* vitellogenesis

Once the ELISA parameters optimized, this method was physiologically validated by detecting changes in VTG levels correlated with the stimulation vitellogenesis induced by squid fractions (*fig.* 7).

Experimental results reveal that an increase in the VTG level can be induced by squid fractions included in the feed in pubescent females. This is especially the case for the hydroalcohol soluble fraction (HAS) fraction and subfractions in terms of VTG concentrations as denoted by the Duncan's new multiple range test after ANOVA (*table* 1). Nonetheless it appears likely that a polar component of the lipid extract has also a positive induction potential. In eyestalk ablated females and in intact females receiving the HAS fraction, the mean VTG concentration rose more than five fold relative to the control levels over the course of 15 days.

In terms of the proportion of individuals responding to the experimental treatments no significant changes were appreciated ($\chi^2 = 5.79$ (N.S.) $p > 0.05$, 7 d.f.).

Table 1. – Results of the ANOVA ($F = 4.01$, $p < 0.05$ with 7 d.f.) and means separation of vitellogenin concentrations (ng/ml) registered in female shrimps after being fed with squid extracts (see *fig.* 7 for abbreviations).

BD	LIP/RF	Lipids	HAS/RF	LIP/PF	HAS/PF	HAS	EABL
0.24	0.34	0.48	0.57	0.59	0.68	0.94	0.98

DISCUSSION

The procedure adopted for VTG purification proved successful in maintaining integral antigenicity and was easily applied when scaling up. Utmost care was devoted to this stage because the quality of the ELISA

relies on this aspect. In fact it is capital for immunisation, calibrating and coating (Ollerich, 1980; Kurstak, 1985).

The specificity of antibodies was established by several lines of evidence. On one hand, the finding of parallel inhibition curves of biological samples which do not deviate significantly suggests the cross-reactivity with close-related structural compounds. On the other hand the absence of reaction with samples from pubescent females and males thus proves that the system is suitable for measuring plasmatic VTG.

It was particularly interesting to find out that the displacement of VTG required a greater quantity of VTL due to its partial recognition by the anti-VTG antibody. This confirms the importance of working in a homologous system in which the same antigen is used to generate antibodies, as a standard and as a competitor, as stated by Nicoll (1975) and Maitre *et al.* (1985). This also evokes the possibility that VTG undergoes some structural changes when entering into the ovary as indicated by Tsumura and Nakagawa (1989), in spite of its qualitative similarity with VTL as reported by Tom *et al.* (1987), Meusy and Payen (1988), Quinitio *et al.* (1989) and many others. Recently Tom *et al.* (1992) mentioned the inconsistence of patterns of VTL immunoprofiles attributable to structural changes taking place in the VTL during oocyte maturation.

Recovery test not only supported specificity but also proved that there was a minimal interference by plasma components in the measurement of immunoreactive VTG.

The reduced values of the coefficient of variation were well within the acceptable limits of the technique and reflected the stability of conditions of the system.

Routine estimations were made with the equilibrium assay because despite its lower sensitivity, it allowed us to determine concentrations over a broader range facilitating in this way the samples dilution and letting us carry out the whole procedure in only 7 h after overnight coating. If desired, sensitivity can be enhanced by using a non-equilibrium assay with an amplification system like PAP or ABC and modifing some of the conditions of the assay (coating concentration, conjugate dilution, etc.).

A number of different techniques have been established to determine VTG levels in crustaceans including immunodiffusion (Tom *et al.*, 1987; Quinitio *et al.*, 1990), electrophoregram scanning (Byard and Aiken, 1984; Nakagawa *et al.*, 1982), RIE (Yano, 1987; Nelson *et al.*, 1988; Mendoza and Fauvel, 1989) and heterogenous ELISA (Derelle *et al.*, 1986; Quackenbush, 1989). Notwithstanding homologous ELISA properly calibrated appears particularly suitable for the analysis of a large number of samples in a rapid, accurate and inexpensive way.

The sensitivity of our technique compares well with other VTG ELISA's for other species (Dumas *et al.*, 1982, Ma *et al.*, 1984, Derelle *et al.*, 1986, Zou *et al.*, 1988, Nuñez *et al.*, 1989, Cuisset *et al.*, 1991).

The relationship between the increase in plasma VTG and ovarian VTL during the period of ovarian growth has previously been reported (Mendoza and Fauvel, 1989; review in Meusy and Payen, 1988), showing that VTG level from a practical standpoint is a valuable indicator of the reproductive state of female shrimp as well as a good predictor. This was confirmed by our experimental results which revealed that it was possible to induce vitellogenesis in very young females without eyestalk ablation and that a component of squid extracts enabled vitellogenesis induction at the same level as with ablation. The exact nature of this factor remains to be elucidated though it seems likely that a component like the mitosis stimulating factor found in the optical glands and haemolymph of squid (Koueta and Boucaud-Camou 1989, 1991) as well as sex steroids from squid gonads (Carreau and Drowsdowsky, 1977; Blanchier *et al.*, 1986) could be at the origin of this activation.

Tom *et al.*, (1992) have noticed changes in the polypeptide pattern of *P. vannamei* VTL during oogenesis a fact which they explain as an increased susceptibility of this protein to enzymatic digestion, being consistent thus with our results.

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