

Extraction systems for isolating esterases having interfacial adsorption

Sistemas de extracción para el aislamiento de esterases con adsorción interfacial

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Abstract

Interfacial esterases present great functional versatility, making them very attractive molecules for industrial applications. The conditions for extracting interfacial esterases previously detected in the sea anemone *Stichodactyla helianthus* and the shrimp *Litopenaeus vannamei* were optimised in this work. Animal homogenates were treated with Triton X-100, Tween 20 and Tween 80 detergents at two different concentrations: critical micellar concentration (CMC) and half of that concentration; 0.5 mol/L NaCl and n-butanol at 5%, 10% and 20% v/v ratios were also tested. Each procedure was compared to the control extraction method using distilled water. The best results were obtained with 20% n-butanol for recovering esterase and phospholipase activity whilst 10% n-butanol extraction was the most effective for lipase activity isolation. This solvent's suitability for isolating interface-activated enzymes could be explained by its ability to dissociate biomolecule aggregates and cause enzyme desorption from the membranes and tissues remaining in the preparation.

Key words: interfacial activation, interfacial esterase, lipase, *Stichodactyla helianthus*, *Litopenaeus vannamei*.

Resumen

En el presente trabajo se optimizaron las condiciones de extracción de esterases con actividad en interfaces, a partir de la anémona marina *Stichodactyla helianthus* y del camarón penecido *Litopenaeus vannamei*. Las esterases interfaciales, cuya presencia en estas especies había sido informada previamente, presentan características funcionales que las hacen muy atractivas para su empleo industrial. Los homogenados de los animales se trataron con los detergentes Tritón X-100, Tween 20 y Tween 80 en dos concentraciones cada

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uno: la Concentración Micelar Crítica (CMC) y la mitad de ésta. Además se empleó NaCl 0,5 mol/L y n-butanol a las proporciones 5, 10 y 20%. Cada variante fue comparada con el método tradicional de extracción con agua destilada, que fue tomado como control. Los mejores resultados se obtuvieron empleando n-butanol al 20%, para recuperar las actividades esterasa y fosfolipasa, y al 10%, en el aislamiento de la actividad lipasa. La efectividad de este solvente en el aislamiento de estas enzimas con afinidad por las interfaces lípido/agua, pudiera estar dada por su capacidad para romper los agregados entre estas moléculas y causar la desorción de las mismas a los restos de membrana y tejidos presentes en la preparación.

Palabras clave: activación interfacial, esterasas interfaciales, lipasas, *Stichodactyla helianthus*, *Litopenaeus vannamei*.

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Introduction

Esterases are enzymes which catalyse the hydrolytic breakdown of ester bonds. Some of them (such as lipases and phospholipases) require lipid/water interfaces to develop maximum catalytic activity (Holwerda *et al.*, 1936; Schonheyder and Volquartz, 1945; Sarda and Desnuelle, 1958; Volwerk *et al.*, 1986; Malcata, 1996; Chahinian *et al.*, 2002; Reis *et al.*, 2009). These enzymes (here called interfacial esterases) are widely spread in nature (Berner and Hammond, 1970; Six and Dennis, 2000) and their functional versatility has conditioned their use in the food, detergents and fine chemical industries (Benjamin and Pandey, 1998; Jaeger and Reetz, 1998; Bastida *et al.*, 1999; Pandey *et al.*, 1999; Segura *et al.*, 2004).

Considering that marine organisms have been less explored than mammals and microorganisms and also because there is a higher probability of finding enzymes having different specificities towards non-natural substrates in marine organisms, the quest for interfacial esterase activity in marine invertebrates has been a major research goal in this field (Nevalainen *et al.*, 2004; Knotz *et al.*, 2006; Park *et al.*, 2008; Perera *et al.*, 2008). Extraction is a critical step in detecting desired enzymatic activity in any living organism. Designing efficient and selective extraction systems ensuring good recovery of such desirable activity and preventing interferences with other enzymes in the assay is a major concern (Mala *et al.*, 2007; Talukder *et*

al., 2007). This is due to insufficient knowledge being available concerning the presence of interfacial esterases in marine invertebrates.

This work describes the extraction of interfacial esterases from the sea anemone *S. helianthus* and the shrimp *L. vannamei*. Animal homogenates were thus treated with Triton X-100, Tween 20 and Tween 80 non-ionic detergents at two different concentrations: CMC and half this concentration (Mogensen *et al.*, 2005). Sodium chloride and n-butanol were also tested. Each procedure was compared to the control extraction method using distilled water.

Interfacial esterases can adsorb on hydrophobic interfaces (Peters *et al.*, 1997; Peters and Bywater, 2001; Berg *et al.*, 2004; Reis *et al.*, 2008a), like those appearing on micellar surfaces (Martinek *et al.*, 1987; Petersen, 1996; Mitra *et al.*, 2005; Shome *et al.*, 2007). Using non-ionic detergents as potential agents for extracting interfacial esterases (Pind and Kuksis, 1988; Iijima *et al.*, 1990; Palomo *et al.*, 2004; Sonesson *et al.*, 2006) thus becomes promising. Increments in ionic strength, though not high enough to reach salting-out-related values, may contribute towards the disaggregation of protein molecules and their desorption from membrane remnants, thereby causing better recovery of these enzymes (Natori *et al.*, 1983; Reers and Pfeiffer, 1987; Ono *et al.*, 1988; Sagiroglu and Arabaci, 2005; Sah and Bahl, 2005). The presence of n-butanol in the extraction medium

may cause the precipitation of other contaminant proteins and may lead to membrane remnant dissociation and esterase disaggregation (Pedersen et al., 2006; Akbar et al., 2007; Koo et al., 2008), enzymes which are very stable in organic solvents (Bornscheuer et al., 1994; Dandavate et al., 2009). Using 20% n-butanol was the best method for extracting interfacial esterases from *S. helianthus* extract.

The latter extraction system's effect (as well as that of the n-butanol proportion) on the specific type of recovered esterase activity was also studied. This was done by comparing esterase, lipase and phospholipase activity. Extracting phospholipase and esterase activities (but not lipase activity) from the *S. helianthus* extract was favoured by 20% n-butanol. This concentration was the best for phospholipase and esterase extraction from *L. vannamei* midgut gland extracts. By contrast, lipase activity from midgut gland extracts was best recovered with 10% n-butanol.

Materials and methods T1

Marine organisms. *S. helianthus* was collected from the coast of Havana by specialists from the National Aquarium of Cuba and from Havana University's Biology Faculty. *L. vannamei* was generously donated by Northwest Biological Research Centre (CIBNOR), Baja California Sur, Mexico. Adult males from culture tanks from the latter species were used during intermolting.

Preparing homogenates. *S. helianthus* homogenates were prepared by mixing whole organisms in a blender, adding 2 ml distilled water per gram animal wet weight at 4 °C. The midgut glands from *L. vannamei* were dissected and homogenised in distilled water using a 1:8 wet weight/volume ratio at 4 °C for 5 minutes. Both homogenates were kept at -20 °C until use.

Extracting interfacial esterases. The following procedures were assessed to study the effect of

different compounds on extracting esterases present in *S. helianthus* homogenates:

- The control method used for comparison, based on adding distilled water to the homogenate;
- Adding non-ionic Triton X-100, Tween 20 and Tween 80 detergents to the homogenates at CMC and half this concentration. Triton X-100, Tween 20 and Tween 80 CMC were 0.2×10^{-3} mol/L, 0.06×10^{-3} mol/L and 0.02×10^{-3} mol/L, respectively, according to the Boehringer Mannheim Biochemical catalogue (1996);
- Adding 0.5 mol/L NaCl to the homogenates (Scopes, 1988); and
- Adding 20% n-butanol at 4 °C to the homogenates (to use high proportions of n-butanol see: Miki *et al.*, 1985; Malik and Low, 1986).

All concentrations and proportions were calculated according to treatment final volume. Each extraction method's product was spun for 1 hour at 15,000 g at 4 °C in a refrigerated centrifuge (Beckman J2-21, USA). Supernatants were dialysed at 1:1,000 extract volume/dialysis solution ratio, first against distilled water and then using a 10 mmol/L sodium phosphate buffer, pH 7.0. Both dialysates were carried out for 6 hours at 4 °C. Extracts were stored at -20 °C until use.

Each treatment was repeated five times. Kolmogorov-Smirnov and Bartlett tests were used for analysing data normality and variance homogeneity, respectively. One-way ANOVA was used for fixed effects model. Means were compared by Duncan multiple ranks test for the extraction study. Student t-test was used for comparing the means between treatments for the study about the effect of the n-butanol-based extraction method on the type of recovered esterase activity (Sigarroa, 1985).

L. vannamei midgut gland homogenates were mixed with n-butanol at 5%, 10% and 20% (Miki *et al.*, 1985; Malik and Low, 1986). Distilled water was used as experiment control. The samples were homogenised for 5 min with constant shaking, centrifuged for 10 min at 10,000 g and the middle layer was collected. This layer was diafiltrated using an ultrafilter with an YM3 membrane (cut-off: 10,000 Da, Diaflo, USA) by spinning for 10 min at 10,000 g and 4 °C. Six washes with distilled water were done to ensure organic solvent removal.

Five replications for each treatment were performed. Kolmogorov-Smirnov and Bartlett tests were used for analysing data normality and variance homogeneity, respectively. Tukey HSD test was used for comparing treatment means (Sigarroa, 1985).

Protein concentration assays. Enzymatic extract protein concentration was determined according to Bradford (1976).

Enzyme activity assays

Esterase activity assay. Esterase activity was assayed from extracts by measuring the amount of *p*-nitrophenol released from *p*-nitrophenylacetate (*p*-NPA) used as substrate. A spectrophotometric continuous method (Spekol 11, Germany) with magnetic stirring was applied. The assay was conducted at a constant temperature (30 °C) in a 1 cm vessel. The reaction mixture consisted of enzymatic extract (0.01 mg/ml final protein concentration) in 0.025 mol/L Tris-HCl buffer (pH 8.0). The reaction system was initiated by adding *p*-NPA (3.85 mmol/L final concentration). Absorbance at 348 nm caused by *p*-nitrophenol release ($\epsilon_{348 \text{ nm}} = 5150 \text{ M}^{-1} \text{ cm}^{-1}$) was monitored. One unit of esterase activity was defined as being the amount of enzyme producing 1 μmol *p*-nitrophenol per minute in assay conditions.

Lipase and phospholipase activity assays. Lipase and phospholipase enzymatic activity were assayed in enzymatic extracts using an automatic titration method (Mettler-Toledo DL-21,

Switzerland) in pH-stat mode. The reactions were carried out with constant stirring and temperature (30 °C). Tributyrin and soybean phosphatidylcholine (PC) were used as substrates for lipase and phospholipase activities, respectively (both Sigma Chemical Co., St. Louis, MO, USA). Substrates were emulsified with arabic gum (Sigma Chemicals Co., USA) in the activity buffer with magnetic stirring for 10 min followed by sonication (Branson 1200, UK) for 15 min. The reaction mixture consisted of enzymatic extract (0.01 mg/ml final protein concentration), 0.14 mol/L emulsified substrate, 3% arabic gum, 0.04 mol/L CaCl₂ in 0.01 mol/L Tris-HCl buffer (pH 8.0). pH was kept constant at 7.0 and 0.01 mmol/L NaOH was used as titrating solution. One unit of lipase/phospholipase activity was defined as being the amount of enzyme which liberated 1 μmol free fatty acids per minute in assay conditions.

Each enzymatic activity was assessed three times. Enzymatic activity was expressed as specific activity (U/mg) by referring to the amount of protein present in the preparations.

Results and Discussion

Effect of different compounds on extracting interfacial esterases

Different extraction methods based on dissimilar principles were compared in this work to identify a procedure enabling the recovery of as much enzymatic activity as possible. The *S. belianthus* sea anemone was taken as a model and the procedures undertaken were assessed by specific esterase activity measurement using *p*-NPA as substrate. This enzymatic activity was chosen as it is the most generally found for the group of enzymes being investigated (Colowick and Kaplan, 1955).

These enzymes are able to adsorb on hydrophobic interfaces (Peters *et al.*, 1997; Peters and Bywater, 2001; Berg *et al.*, 2004; Reis *et al.*, 2008a), like those appearing on biological membrane surfaces and detergent micelles

(Martinek *et al.*, 1987; Egmond 1996; Mitra *et al.*, 2005; Shome *et al.*, 2007). Considering this property, three non-ionic detergents were used as candidate extraction systems (Triton X-100, Tween 20 and Tween 80); they were used at two concentrations: CMC and half this concentration (Mogensen *et al.*, 2005), totalling six extraction methods tested. Only Triton X-100 at CMC was significantly better than the control method (based on distilled water) (Figure 1).

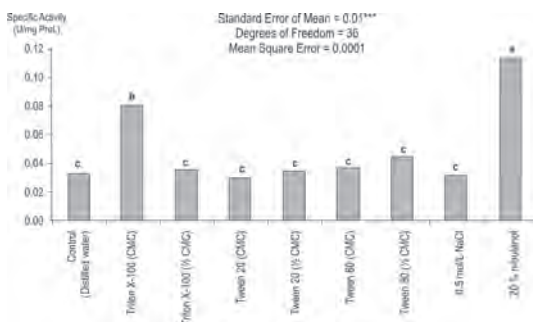


Figure 1. Comparing different methods for extracting interfacial esterases from *S. belianthus* through specific esterase activity assessment with *p*-NPA. Each treatment was performed five times. Means were compared by Duncan multiple ranks test. Different letters show significant differences ($p < 0.01$).

The fact that it was necessary to reach CMC (Mogensen *et al.*, 2005) to obtain good enzyme recovery with Triton X-100 could be explained by the high affinity for lipid/water interfaces exhibited by these molecules (Sonesson *et al.*, 2006; Reis *et al.*, 2008b). The Triton X-100 detergent's chemical structure also seems to have had a positive influence on extraction (Isobe and Sugiura, 1977; Natori *et al.*, 1983; Palomo *et al.*, 2004; Ras *et al.*, 2008) when comparing Tween 20 and Tween 80 (Figure 1).

Adding 0.5 mol/L NaCl to the homogenates was no better than control method as an attempt to overcome enzyme aggregation and increase solubility (Natori *et al.*, 1983; Reers and Pfeiffer, 1987; Ono *et al.*, 1988; Pind and Kuksis, 1988; Sah and Bahl, 2005). However, working at low temperatures (4 °C) and in the presen-

ce of 20% n-butanol in the extraction medium was the best strategy when esterase activity was monitored with *p*-NPA assay (Figure 1). This probably resulted from this solvent's disaggregation effect (Montero *et al.*, 1993; Snellman *et al.*, 2002; Palomo *et al.*, 2003) on interactions amongst interfacial esterases and between them and the membrane remnants present in the sample (Pedersen *et al.*, 2006; Koo *et al.*, 2008). n-butanol can also produce accompanying proteins' precipitation, thereby increasing specific activity (Akbar *et al.*, 2007) compared to other non-precipitating methods. On the other hand, Bornscheuer *et al.* (1994) and Dandavate *et al.*, (2009) have reported interfacial esterases' great stability in organic solvents.

Even though Triton X-100 at CMC and n-butanol at 20% and 4 °C procedures were the most promising, it was decided to continue the study using the organic solvent extraction method. This criterion was influenced not only by the fact that the latter method was able to recover the highest specific enzymatic activity values (Figure 1) but also because it seemed more attractive considering the purposes of this work. Indeed, from a practical point of view, organic solvent extraction is a wise option. It is well known that post-extraction detergent removal is not always effective (Zhong *et al.*, 2006) and on many occasions requires chromatographic steps which do not ensure complete elimination and place a negative burden on process cost (Furth, 1980; Robinson *et al.*, 1984). However, it is perfectly possible to achieve organic solvent removal through exhaustive dialysis or ultrafiltration (Luisi and Laane, 1986).

The effect of the n-butanol-based extraction method for interfacial esterases on the type of esterase enzymatic activity recovered

The most particular type of enzymatic activity potentially recoverable using n-butanol was explored once the treatment for extracting the highest specific esterase activity from *S. belianthus* homogenates had been established. The

n-butanol treatment was accordingly assessed via the following specific activities: esterase, lipase and phospholipase, using *p*-NPA, tributyrin and soybean PC as substrates, respectively.

Figure 2 shows that n-butanol treatment was able to isolate enzyme quantities having esterase and phospholipase activity per milligram of whole proteins which was significantly higher than that obtained using the control method. Nevertheless, lipase activity was not favoured by the afore-mentioned treatment.

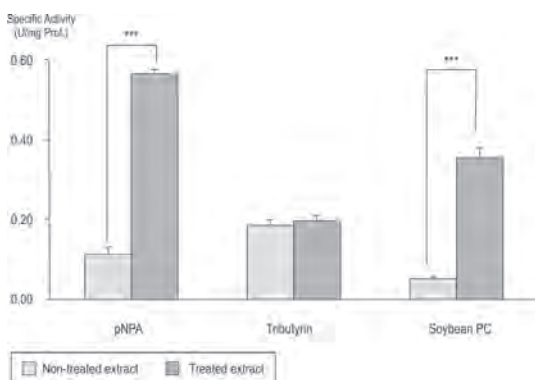


Figure 2. Comparing different enzymatic activities recovered by the extraction method for interfacial esterases from *S. helianthus* based on n-butanol at 20% and 4 °C, by assessing the following specific activities: esterase, lipase and phospholipase, with *p*-NPA, tributyrin and soybean PC as substrates, respectively. Student's t-test was used for comparing the means for each control-treatment pair. Three asterisks indicate significant differences ($p < 0.001$). Error bars indicate mean standard deviation for five replications.

Table 1. Purification of esterase, lipase and phospholipase enzymatic activities, assayed with *p*-NPA, tributyrin and soybean PC, respectively, through the extraction method for interfacial esterases from *S. helianthus* based in n-butanol at 20% and 4 °C.

	Non-treated extract (distilled water)	Treated extract (20% n-butanol and 4 °C)		
Substrates	-	<i>p</i> -NPA	tributyrin	Soybean PC
Yield (%)	100	443	93	595
Purification factor (fold)	1	5	1.05	6.8

The treatment resulted in higher than 100% yield for both phospholipase and esterase activity, compared to the control method. Purification factors in both cases showed the process' semi-purification nature since this method was able to support these enzymes' selective extraction. Both criteria manifest this method's better application for phospholipase activity recovery (Table 1).

Effect of n-butanol proportion in the extraction medium on the type of esterase enzymatic activity recovered

The effect of organic solvent proportion on the type of esterase enzymatic activity recovered from *L. vannamei* midgut glands was also studied. Three distinct conditions were tested in the experiment: n-butanol at 5%, 10% and 20%. Extracts were assessed for esterase, lipase and phospholipase specific enzyme activity, with *p*-NPA, tributyrin and soybean PC as substrates, respectively.

A previous study (data not shown) has shown that extract clarification by spinning organ homogenates and subsequently collecting the middle phase (before n-butanol treatment) has promoted the loss of phospholipase activity. On the contrary, this activity remained when assayed in non-clarified extracts. Non-clarified *L. vannamei* midgut glands homogenates were thus submitted to n-butanol treatment (an already established procedure for *S. helianthus*).

Some authors have recommend using 20% n-butanol at 4 °C as the extraction variant attaining the greatest values for membrane-associated enzyme recovery (Ayala *et al.*,1985; Luisi and Laane, 1986). Increments in esterase activity were observed with increasing n-butanol proportions when extracts were submitted to enzymatic assays with *p*-NPA. Extracting this enzymatic activity was not favoured by n-butanol at the smallest of the proportions used here (Figure 3).

A bell-curve like behaviour was detected for the lipase activity against n-butanol percentage in the extraction medium when this activity was assayed with a more lipase-specific substrate such as tributyrin. The highest specific activity was thus extracted with 10% n-butanol; however, positive effects were not observed at the other studied proportions (Figure 4).

This result suggests a loss of stability for the lipolytic enzymes (being components of this fraction) at n-butanol proportions higher than 10%. Thereafter, 10% was the most effective n-butanol proportion for isolating lipases from *L. vannamei* midgut gland, a rule which did not apply to other esterases extracted with higher efficiency at 20% of organic solvent (Figure 4).

Recovering enzymatic molecules having phospholipase activity was solely possible

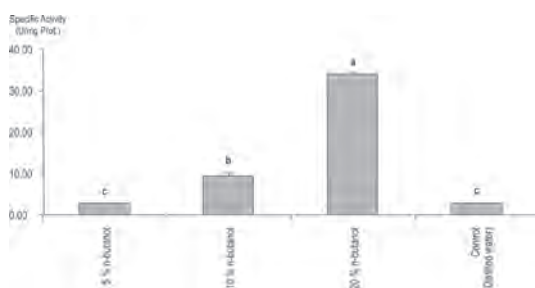


Figure 3. Comparing different n-butanol proportions in the extraction medium regarding their capacity for esterase enzymatic activity recovery from *L. vannamei* midgut gland through specific esterase activity assessment with *p*-NPA. Different letters indicate significant differences ($p < 0.05$), according to Tukey HSD test. Error bars indicate mean standard deviation five replications.

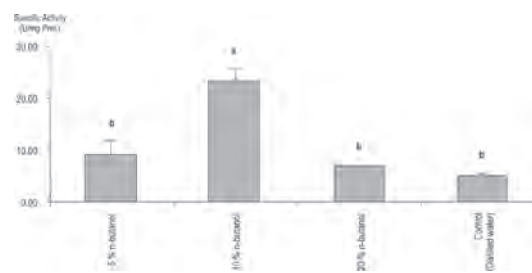


Figure 4. Comparing different n-butanol proportions in the extraction medium regarding their capacity for lipase enzymatic activity recovery from *L. vannamei* midgut gland through specific lipase activity assessment with tributyrin. Different letters indicate significant differences ($p < 0.05$), according to Tukey HSD test. Error bars indicate mean standard deviation for five replications.

when 20% n-butanol was used (Figure 5). This result indicated that these enzymes removal from membrane and tissue remnants, as well as from the lipid/water interface generated during manipulation, could only be facilitated at 20% n-butanol concentration (from all tested values). It is worth noting the absence of enzymatic activity in the control method (Figure 5). This result suggested the presence of phospholipases in the *L. vannamei* midgut gland which showed higher affinity for hydrophobic interfaces than the phospholipases present in

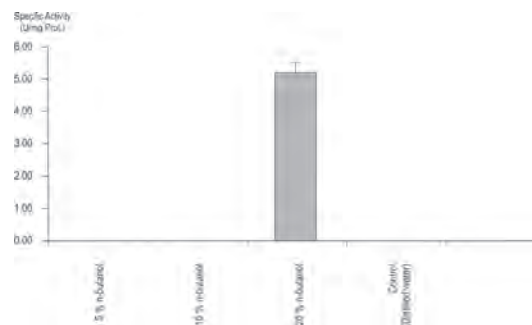


Figure 5. Comparing distinct n-butanol proportions in the extraction medium regarding their capacity for phospholipase enzymatic activity recovery from *L. vannamei* midgut gland via specific phospholipase activity assessment with soybean PC. Error bar indicates mean standard deviation for five replications.

S. helianthus. The latter enzymes' activity was detected in the extracts obtained using the control method (Figure 2).

Conclusions

The results point to the method for extracting interfacial esterases from *S. helianthus* based on 20% n-butanol at 4°C as being the best procedure out of the nine tested here when the process was evaluated by assessing specific enzymatic activity with *p*-NPA. Using Triton X-100 non-ionic detergent at CMC was also promising, although the recovered specific activity was lower for the detergent when compared to the organic solvent procedure. 20% n-butanol treatment enhanced the extraction and semi-purification of phospholipase and esterase activities, especially favouring the former's recovery and not exerting any influence on lipase activity. Higher esterase specific activity values were recovered from *L. vannamei* midgut gland with increased n-butanol percentage (5%-20%), assayed with *p*-NPA. On the contrary, optimal organic solvent percentage for extracting esterases having lipolytic activity in this specie was 10%, suggesting functional damage for these enzymes at higher n-butanol proportions. Extracting molecules having phospholipase activity was only successful with 20% n-butanol and measurable activity was not detected, even when the control method was used. This finding suggests the presence of phospholipases having greater affinity for lipid / water interfaces in *L. vannamei* midgut gland than in *S. helianthus*.

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