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Short communication

Elements for optimizing a one-step enzymatic bio-refinery process of shrimp cuticles: Focus on enzymatic proteolysis screening

R. Baron[a,*], M. Socol[a], R. Kaas[b], A. Arhaliass[c], J. Rodriguez del Pino[a], K. Le Roux[d], C. Donnay-Moreno[a], J.P. Bergé[a,1]

[a] Ifremer, BRM, Rue de l'Ile d'Yeu, BP21105, 44311 Nantes cedex 03, France
[b] Ifremer, PBA, Rue de l'Ile d'Yeu, BP21105, 44311 Nantes cedex 03, France
[c] Université de Nantes, GEPEA UMR CNRS 6144, CRTT, Boulevard de l'Université, 44600 Saint-Nazaire cedex, France
[d] Ynsect, Rue de l'Ile d'Yeu, BP21105, 44311 Nantes cedex 03, France

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ABSTRACT

This article complements an earlier work published in 2015 Baron et al. (2015) that showed the interest of a shrimp shells bio-refining process. We compare here the effect of eleven commercial proteases at pH 3.5 or 4.0 on a residual amount of shrimp shells proteins after 6 h at 50 °C. The two pH are obtained when respectively 40 and 25 mmol of formic acid are added to 5 g of mild dried shell. Deproteinisation yield above 95% are obtained. Residual amino acids profile in the solid phase was identical for the eleven proteases except for pepsin which was similar to the raw material profile. A significant relative increase in the proportion of Glycine is observed for the ten other cases. Likewise, shapes of size exclusion chromatograms of the dissolved phase are similar except with pepsin.

1. Introduction

Purification of crustacean chitin shells has been studied by many authors [1–5,8–22] [6,7] and today represents an important economic activity particularly in the context of shrimp shells value-enhancing schemes [23]. In fact the applications of chitin and its derivatives are more and more widespread. However, the process used is purely chemical and allows only an enhancing value of a small portion of the biomass. Efforts were therefore made to limit the use of chemicals and make this type of purification more sustainable. Bio-refining of crustacean shells, especially shrimp, is an economic, technical and scientific objective already described by some authors [1,2,4,5,10,15–17,20,22] [6,7]. Two biotechnological ways are found in literature: fermentation [5,10,16,17] or enzymatic hydrolysis [1,2,4,15,16,20,22] [6,7]. A bio-refining process in a single step by an exogenous proteolysis in acidic media would enable us to perform chitin purification and deproteination in the same time. Recently, we have shown [1] the promising potential of the bio-refining in a single step of *Litopenaeus vannamei* shrimp shells. The authors have mainly focused on the kinetics of demineralization and the choice of a suitable acid that could ensure a high demineralization yield (>98%) for a pH value close to 4.0 (classical preservation value). Formic acid best fits the selected target criteria. This acid achieves a demineralization yield of 99% at pH 3.5 and 98% at pH 4.0, depending on the selected volume. An increase in solution volume promotes final demineralization. In 6 h, a combination of formic acid and ASP enzyme (Acid Stable Protease), in sufficient concentration, allowed to go beyond the 95% protein removalyield, at pH 3.5 or 4.0. The purity of the obtained chitin is respectively 92% at pH 3.5 and 90% at pH 4.0. The resulting chitin purity over 90%, for a single stage process working in 3.5–4 pH range avoids the additional steps of neutralization of both the solid and dissolved phases.

Here we focus on determining the effectiveness of ten other commercial proteases compared to the ASP enzyme working in 3.5–4.0 pH range. The determination of an enzyme reaching a maximum deproteination yield after 6 h of hydrolysis in 3.5–4.0 pH range, and preferably at pH 4.0 needing less amount of acid, was first sought. The amount of residual proteins was determined using the sum of the quantitative analysis of 16 amino acids. The amino acid profile was also analyzed. The study of size exclusion chromatographs in conjunction with the molecular weight distribution of the generated peptides was conducted on the

* Corresponding author.
E-mail address: rbaron@ifremer.fr (R. Baron).
[1] Present adress: Idmer, 2 Rue Batelière, 56100 Lorient.

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dissolved phase. All information collected will provide substantial support for the choice of the enzyme.

2. Materials and methods

2.1. Raw material

The raw material used here corresponds to the *Litopenaeus vannamei* shrimp exoskeleton thawed, peeled by hand, dried, crushed and sieved. The size of the pieces of shell was between 0.5 and 1.0 mm. The protocol for obtaining the raw material is described in the previous article [1].

Composition of the ground cuticle, after mild drying, was: 11.2 ± 2.0% water, 23.4 ± 3.6% minerals (∼1.17 g), 35.0 ± 2.0% proteins (∼1.75 g), 25.2 ± 3.0% chitin (∼1.26 g), and ∼5% others (fatty acids, glycosides, pigments). Composition in brackets are given for 5 g of dried raw material.

2.2. Characterization of materials

Ash content was measured gravimetrically, percentages of residual minerals (RM) and demineralization yield (DY) calculated as described in Baron et al. [1]. Protein content is obtained by summing the concentrations of 16 amino acids which were identified, percentages of residual proteins (RP) and deproteination yield (PY) were calculated according to Baron et al. [1].

2.3. Experimental setup and samples preparation

For experiments, a fixed initial weight of 5.0 g of mild dried shrimp cuticles was used in a preset volume of acid solution (150 mL) under constant continuous stirring (300 rpm) with magnetic stirrers. Temperature was controlled at 50 °C with thermostatic plates.

Each time point corresponded to a specific test with 5.0 g of cuticle and the whole reaction volume (solid and liquid phases) was collected to ensure the consistency and accuracy of the results. All the solids were removed by filtering with Nylon filters of mesh size 300 μm. Reaction on solids was stopped by rinsing abundantly with 500 mL of distilled water.

Formic acid was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

The molar ratio needed to obtain a desired pH value at 50 °C is estimated, in a first approximation, by Henderson's relation (calcium carbonate representing more than 90% of minerals [1]).

$$K_a = \frac{[HCOO^-]\,[H_3O^+]}{[HCOOH]} \text{ and } pH = pK_a + \log_{10}\left(\frac{[HCOO^-]}{[HCOOH]}\right) \text{ with pKa} = 3,75 \text{ at } 2^\circ C$$

Solution pH was measured with an analytical pHmeter (CyberScan pH/Ion 510, Eutech Instruments) and with an electrolytic pH electrode (InLab pro expert, Mettler Toledo).

2.4. Enzymes

Enzyme activities are either not identical, or expressed in different units, or not supplied by the manufacturer. This makes it difficult to determine the amount of enzyme to be added in order to carry out this comparative work. We have chosen to work with a sufficient amount of enzyme with a weight to weight ratio of enzyme/proteins of 25%.

For 5 g of shell, 1.75 g of proteins is assumed to be present (see Section "raw material"). 437.5 mg of enzyme (=25% of 1.75 g) are added 5 min after shells were poured in 150 mL reaction volume.

2.5. Weight distribution analysis of peptides generated after hydrolysis

Twenty milligrams of lyophilized aqueous phase samples from the hydrolysates were eluted in 10 mL solvent: 30% acetonitrile/0.1% trifluoroacetic acid, and were then centrifuged at 10,000*g* during 10 min in a Beckman Coulter Avanti J-25 refrigerated at 10 °C. The sludge and the soluble fraction were then separated [24].

Peptides molecular weight distributions of the soluble fraction were determined by gel filtration chromatography on a FPLC Superdex Peptide 10/30 GL column (Pharmacia Biotech): exclusion size range of 100 − 7.000 Da, eluting solvent (previously defined). The flow rate was 0.5 mL/min. Detection signal was performed with a Diode Array Detector DAD Shimadzu SPD M20A. Detection of peptide bonds was preferentially measured at an absorbance of 205 nm. Standards injected were Glycine: Gly (72 Da), Gly–Gly (132 Da), Gly-Gly–Gly (189 Da), Gly-Gly-Gly-Gly–Gly (303 Da), Leupeptin (463 Da), Substance P (900 Da), Neurotensin (1673 Da), Insulin Chain B (3496 Da), Aprotinin (6511 Da).

A calibration curve between retention time and peptide weight was established using standard peptides in triplicates.

3. Results and discussions

The relation between molar ratio and experimental pH value after 6 h at 20 °C was sketched in [1]. On this basis and using the

Table 1
Enzyme characteristics and properties.

Enzyme	Micro-organism or other	Type of enzyme	Society	Optimal pH[a] (pH range)	Optimal T[a] (T range)
DP 401 2100 SAPU/g	–	Acid fungal protease	Valley Research (DSM)	3 (2–4)	45–50
DP 404 542000 HUT/g	–	Acid fungal protease	Valley Research (DSM)	4 (2,5–6,5)	50–55
Fungal Protease 500000 HU/g	Aspergillus oryzae	–	Bio-cat	3 (3–6)	50 (25–60)
Izyme BA 0.15 AU/g	–	Aspartate protease	Novozyme	3 (2–4)	50
FPE EPP 003	–	–	DSM	(3–5)	35
–					
Protex 15 L 1000 SAPU/g	Trichoderma reesei	–	Genencor	(4–5)	
Protex 26 L 2000 SAPU/g	Aspergillus niger	–	Genencor	(3,5–4,5)	
Sumizyme AP-L 2000 U/g	Aspergillus niger	Endopeptidase	Shin Nihon Chemical Co	3 (3–5)	60 (50–60)
Prolyve Pac	Aspergillus niger	–	Lyven	3 (2–4)	60 (50–65)
–					
ASP 3000 SAPU/g	Aspergillus niger	–	Bio-cat	2,5 (2–3,5)	30–60
Pepsin 56000 U/g	Gastric mucosa	Peptidase	Sigma-Aldrich	2 (2–4)	37 (30–50)

- not defined.
[a] for a specific substrate mentioned in their technical document.