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Production of tuna waste hydrolysates by a commercial neutral protease preparation

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Abstract

The fish protein hydrolysates (FPH) are of increasing interest due to their potential applications as a source of bioactive peptides or as nitrogenous substrates for the fermentation media. A waste product from the canning industry was hydrolysed using a commercial protease "Umamizyme". Enzymatic hydrolysis was investigated in a 1-l batch reactor at pH 7 and 45 °C. The influence of the process variables (enzyme/protein substrate ratio ranging from 0.1 to 1.5% (w/w) protein) was studied with regards to the extent of the proteolytic degradation, the nitrogen released and the molecular weight distribution of the peptides. A degree of hydrolysis up to 22.5% was obtained with an enzyme/substrate ratio of 1.5%, after 4 h of hydrolysis. A linear correlation was found to exist between the hydrolysis degree and the nitrogen recovery. The limiting factor of hydrolysis was deduced from a set of experiments conducted at 45 °C and pH 7 where the effects of intermediate substrate and enzyme addition were studied. Results showed that the protease "Umamizyme" performed as effectively as Alcalase® 2, 4L for the tuna waste solubilisation. However, the Umamizyme stability is lower than Alcalase® 2, 4L.
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1. Introduction

Recognition of the limited biological resources and increasing environmental pollution have emphasised the need for better and more value-added utilisation of the under-utilised fish and the by-products from the fishing industries. Traditionally, much of this material has been converted to powdered fish meal by a combined process of cooking, separation of soluble from insoluble, concentration of the soluble and dehydration of the insoluble [1]. Enzymatic hydrolysis is an alternative approach to recovering biomass from marine origin and the result is a soluble product known as fish protein hydrolysate, or FPH. The soluble hydrolysate is subjected to dehydration, resulting in a more stable, powdered form with a high protein content [2].

As total world tuna catches are about 3 billion metric tons [3], canned fish processing generates solid wastes that can be as high as 50−70% of the original raw material. Within the viscera, tuna stomach representing 1.5% of the tuna total weight is a rich source of nutritionally valuable proteins with a well-balanced essential amino acid composition. Preparation of tuna waste hydrolysate would provide means for better utilisation of the by-products, traditionally used for pet-food. Uncontrolled or prolonged proteolysis will result in the formation of highly soluble peptides exhibiting useful nutritional properties, but generally

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devoid of the functional properties associated with native protein. Through the control of the process parameters such as pH, time and enzyme/substrate ratio, it is possible to produce hydrolysates whose components may present some interesting properties. The peptides so obtained may be used in a variety of applications including the search of biologically active peptides. Recent works showed that the hydrolysis of tuna or sardine and cod by-products using Alcalase® 2, 4L in controlled conditions provided hydrolysates in which hormone-like peptides and growth factors were detected [4–7]. These hydrolysates have also the potential for being the source of peptones for microbial growth [8,9]. However, the whole fish by-products of protease-assisted hydrolysis are mainly evaluated for their nutritional and functional properties as shown by many papers published on fish proteins such as capelin [10], sardine [11], lobster [12,13], cod [1] and Pacific whiting [14].

Many authors, in view of the economic interest in the recovering of protein from poorly studied species of fish have compared some commercial proteases in order to test the most suitable one for the substrate employed. The most common commercial proteases reported (in the hydrolysis of fish protein) are both from plant source such as papain [10,11,15,16] or from animal origin such as pepsin [13] and chymotrypsine or trypsine [17]. Enzymes of microbial origin have been also applied to the hydrolysis of fish. In comparison to animal- or plant-derived enzymes, microbial enzymes offer several advantages including a wide variety of available catalytic activities, greater pH and temperature stabilities [2]. Generally, Alcalase® 2, 4L-assisted reactions have been repeatedly favoured for fish hydrolysis due to the high degree of hydrolysis that can be achieved in a relatively short time under moderate conditions compared to neutral or acidic enzymes [14,16,18–20].

The impact of the enzyme's specificity is a key factor influencing both the characteristics of hydrolysates and the nature and composition of peptides produced. Proteolysis can operate either sequentially, releasing one peptide at a time, or through the formation of intermediates that are further hydrolysed to smaller peptides as proteolysis progresses, which is often termed "the zipper mechanisms" [21]. Depending on the specificity of the enzyme, environmental conditions and the extent of hydrolysis, a wide variety of peptides will be generated. The resultant protein hydrolysate will possess peculiar properties according to the new peptides generated. The variety of food-grade proteolytic enzymes is wide and offers enzymologists good opportunity to produce fish waste hydrolysates.

In a recent paper [8], we investigated the tuna waste hydrolysis using Alcalase® 2, 4L and we shown that the concentration of hydrolysable bonds was one of the main factors controlling the hydrolysis rate. Otherwise, we performed in our lab an initial survey of several neutral and basic proteases on tuna waste solubilisation. The results indicated that the preparation Umamizyme (peptidase 723) from a mold, *Aspergillus oryzae* (a product of Amano Pharmaceutical Ltd., Japan) looked promising from both combining a good yield of hydrolysis and a high proportion of soluble peptides in the desired range of size i.e. below 2000 Da (Data not published). According to the indications provided by Amano Enzyme Europe Ltd., Umamizyme has high peptidase activity in contrast to other fungal proteinase preparations and the proteolytic combination system (peptidase and proteinase activities) is possible to hydrolyse various proteins at high level. It seemed interesting to compare the mechanism of action of these two proteases Alcalase® 2, 4L and Umamizyme for the solubilisation of a same substrate.

The following presents the results of a study evaluating the enzyme Umamizyme for its ability to solubilise ground tuna stomach. The principal characteristics of interest were the degree of hydrolysis (DH (%)) and the molecular weight range of the peptide produced, as well as the limiting factors influencing the hydrolysis rate.

2. Experimental

2.1. Substrate

Yellowfin tunas (*Thunnus albacares*) caught in the Indian Ocean and immediately frozen once on board were obtained from a fish processing company "Socoprex" (Concarneau, France). The stomachs were taken from the frozen fish. Heat inactivation of endogenous stomach enzymes (100 °C, 20 min) was carried out prior to pH adjustment and addition of protease in order to denaturate the endogenous

stomach enzymes, mainly the pepsin. All reagents used were of analytical grade.

2.2. Enzyme

"Umamizyme", an enzyme prepared from a strain of *A. oryzae*, was a gift from Amano Pharmaceutical Ltd., Japan. The food-grade enzyme, having a specific endopeptidase activity of 31,100 U/g and exopeptidase activity of 74.8 U/g was stored at 4 °C until it was used for the hydrolysis experiments. Its optimum activity occurs at a temperature of 45 °C.

2.3. Preparation of the hydrolysate

Hydrolysis experiments were carried out in a 1-l thermostatically stirred-batch reactor using the pH-stat method. The reaction flask contained ground tuna stomach and deionised water making up an initial substrate concentration (S (w/w)) of 11% crude protein ($N \times 6.25$). All reactions were performed at pH 7 and 45 °C following the instructions of maximum activity and stability of the enzyme provided by Amano Enzyme Europe Ltd. The hydrolysis conditions (pH, temperature, enzyme concentration and stirring speed) were controlled over the time. The enzyme/protein substrate ratios were in the 0.1–1.5% (w/w protein) range. Control experiments were performed without enzyme addition. All experiments were carried out in triplicate.

An initial 15 min mixing was done for the adjustment of pH, through the addition of NaOH 2N, and temperature to the desired value. The enzyme was then added and the reaction allowed to proceed for 4 h under constant agitation at 500 rpm. The volume of NaOH solution needed to keep the pH constant during digestion was recorded to allow calculation of degree of hydrolysis (DH). Reactions were terminated by heating the solution to 95 °C for 20 min, assuring the inactivation of the enzyme. The resulting slurry was centrifuged at 22,000 × g for 15 min.

2.4. Determination of the degree of hydrolysis

Degree of hydrolysis (DH) was generally used as a proteolysis monitoring parameter when the pH-stat method was employed. The pH-stat reaction allowed the estimation of DH based on the consumption of alkali to maintain a constant pH at the desired value [19].

The values for DH could be determined using the following equation described by Adler-Nissen [22–24]:

$$\mathrm{DH}\,(\%) = \frac{BN_\mathrm{b}}{M_\mathrm{p}\alpha h_\mathrm{tot}} \times 100$$

where DH was the percent ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied (h_tot). The value of h_tot was estimated as the sum of the mmol of each individual amino acid per g protein ($N \times 6.25$), this being found by determining the amino acid composition of the proteic substrate [25]. The variable B was the amount of alkali consumed to keep the pH constant during the reaction, N_b was the normality of the alkali, M_p was the mass of the substrate (protein, determined as $N \times 6.25$) in the reaction and α was the average degree of dissociation of α-NH_2 groups released during hydrolysis.

2.5. Size exclusion chromatography

Molecular weight distribution of peptides in the different hydrolysates was determined by gel permeation chromatography on a Superdex Peptide HR 10/30 column (Pharmacia/LKB) according to the procedure described by Guérard et al. [8]. Peptides of known molecular weight (Sigma) were used to calibrate the column. A relationship between the retention time and the log of the molecular mass of peptides used as standards was established. In the different hydrolysates, peptides were sorted in 5 fractions covering the range of 0−500 Da (fraction V), 500–1000 Da (fraction IV), 1000−2000 Da (fraction III), 2000−5000 Da (fraction II) and above 5000 Da (fraction I). The relative areas of each fraction were given in percent of the total area.

2.6. Nitrogen recovery (NR)

NR was used as an index of nitrogen solubilization to describe the hydrolysis yield. After the hydrolysis reaction, the soluble fraction was separated from the non-soluble fraction by centrifuging at 22,000 × g for 10 min. The total nitrogen in the soluble fraction and the total nitrogen in the hydrolysate were determined

using the Kjeldahl method (AOAC, 1990). NR was calculated according to Benkajul and Morrissey [14] using the following equation:

$$\mathrm{NR}\ (\%) = \left[\frac{\text{total nitrogen in supernatant (\%)}}{\text{total nitrogen in the hydrolysate (\%)}}\right] \times 100$$

2.7. Detection of residual proteolytic activity

The residual proteolytic activity was determined according to the Nielsen and Kirkegaard procedure [26]. A sample of protein hydrolysate was added into a 2-mm hole in gel containing casein, *N*,*N*-dimethyl casein and gelatin. Any dissolved active protease diffusing into the gel and hydrolysing casein will produce a circular disc-shaped opaque zone, clearly visible against non-degradable gel due to the precipitation of calcium caseinate. The image analysis consisted in Gel Doc 1000 system combining a darkroom, a UV transilluminator workstation, a CDD camera and the Quantity One software (Bio-Rad). The result was expressed as a reduction of activity compared to the enzyme dosage used in production.

3. Results and discussion

3.1. Effect of enzyme substrate ratio on the degree of hydrolysis

The value h_{tot} determined in this work for tuna stomach proteins, 6.245 meq (g $N \times 6.25$) is lower than the one given by the Novo manufacturer [27] for fish muscle:7.3 meq (g $N \times 6.25$) and the one evaluated by Diniz and Martin [19] for dogfish muscle: 7.2 meq (g $N \times 6.25$).

During the hydrolysis, fish tissues were rapidly converted from viscous mince into a free flowing liquid. Hydrolytic curves of tuna stomach by Umamizyme were reported in Fig. 1. All curves exhibited an initial fast reaction followed by a slowdown. The shape of these progress curves was similar to that reported for enzymatic hydrolysis of different protein

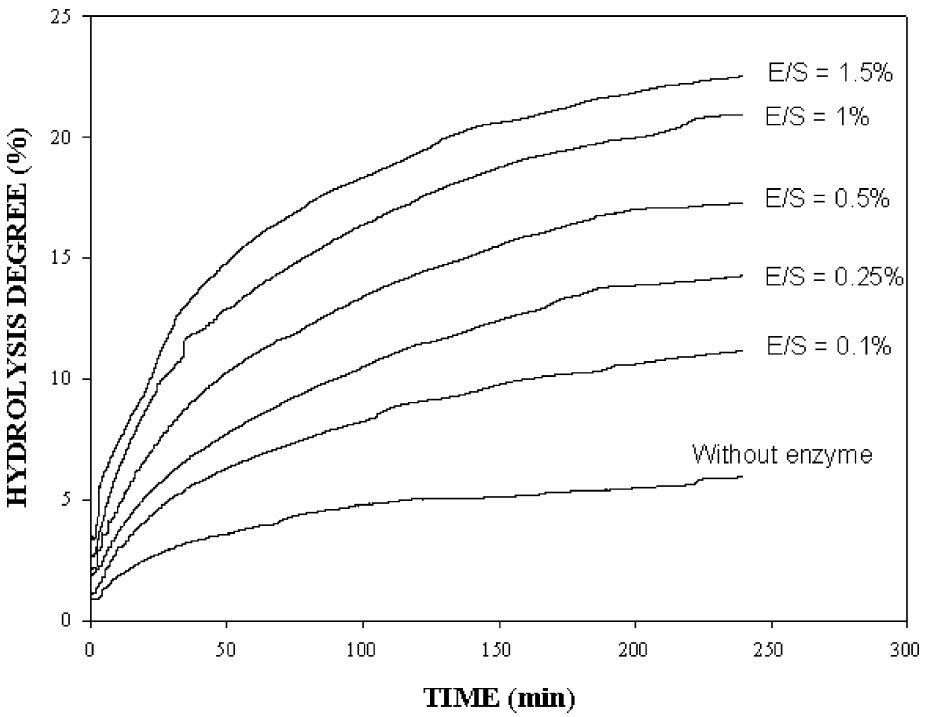

Fig. 1. Effect of enzyme/substrate ratio (w/w protein) ranging from 0.1 to 1.5% on the degree of hydrolysis at 45 °C and pH 7.

substrates such as fish [10,11,25], chickpea [28], meat by-products [29], sunflower [30]. With regards to the effect of the enzyme concentration, it was found that the hydrolysis degree increased with higher enzyme concentrations. A final DH up to 22.55% was achieved within a 4-h incubation with Umamizyme at a 1.5% *E*/*S* (w/w protein) ratio. Less important increases were found with enzyme treatment at concentration above 1%. Using the same substrate, the Umamizyme hydrolysates presented final DH values as high as those obtained using Alcalase® 2, 4L at concentrations ranging from 5.6 to 85 AU/kg raw material [4].

When $\log_{10}$ (enzyme concentration) versus DH were plotted, linear relationships were observed (Fig. 2). This result was in agreement with Benkajul and Morrissey [14]. These authors reported the existence of a linear relationship between the $\log_{10}$ (enzyme concentration) and DH for enzymatic hydrolysis of Pacific whiting using Alcalase® 2, 4L and Neutrase. These authors also suggested that, from this relationship, the exact concentration of enzymes required to hydrolyse the substrate to a required DH could be calculated. Diniz and Martin [19] used surface methodology to study the effects of pH, temperature and enzyme/substrate ratio on the degree of hydrolysis of Dogfish proteins. The polynomial model they proposed was well adjusted to the experimental data and was sufficiently accurate for predicting the DH for any combination of independent variables within the ranges studied.

3.2. Peptide molecular range

Size exclusion chromatograms of hydrolysates at different DHs are shown in Fig. 3. Samples were analysed after a 4-h hydrolysis. From the chromatographic data, we observed that all hydrolysates were composed of low molecular weight peptides.

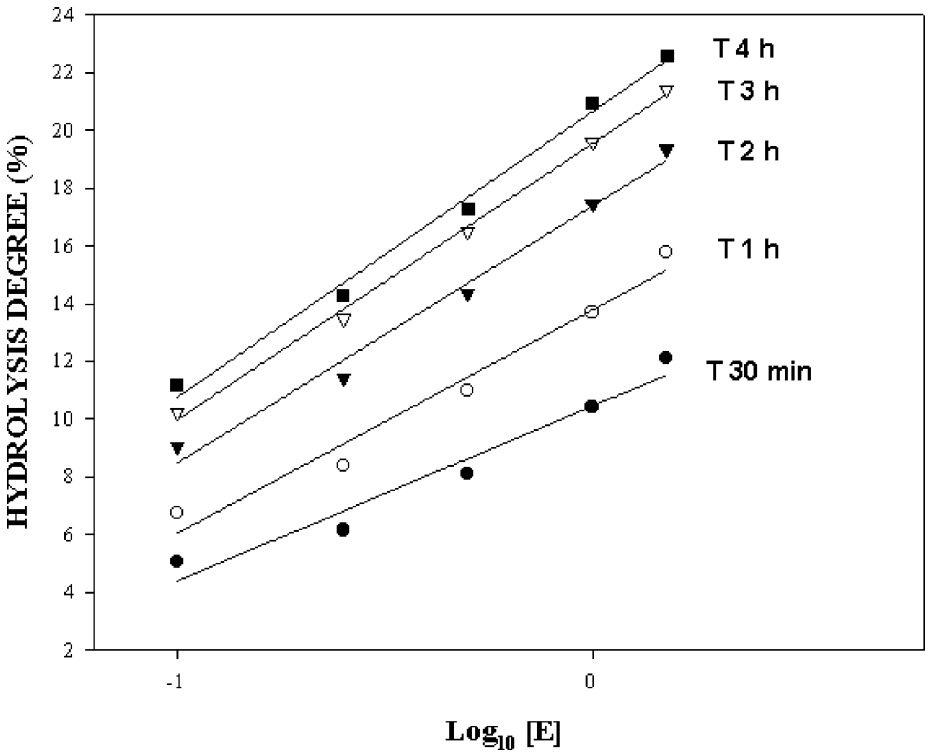

Fig. 2. Relation between $\log_{10}$ (enzyme concentration) and DH for tuna stomachs treated with "Umamizyme". The hydrolytic reaction was run for 30 min, (●); 1 h, (○); 2 h, (▼); 3 h, (▽) and 4 h (■).

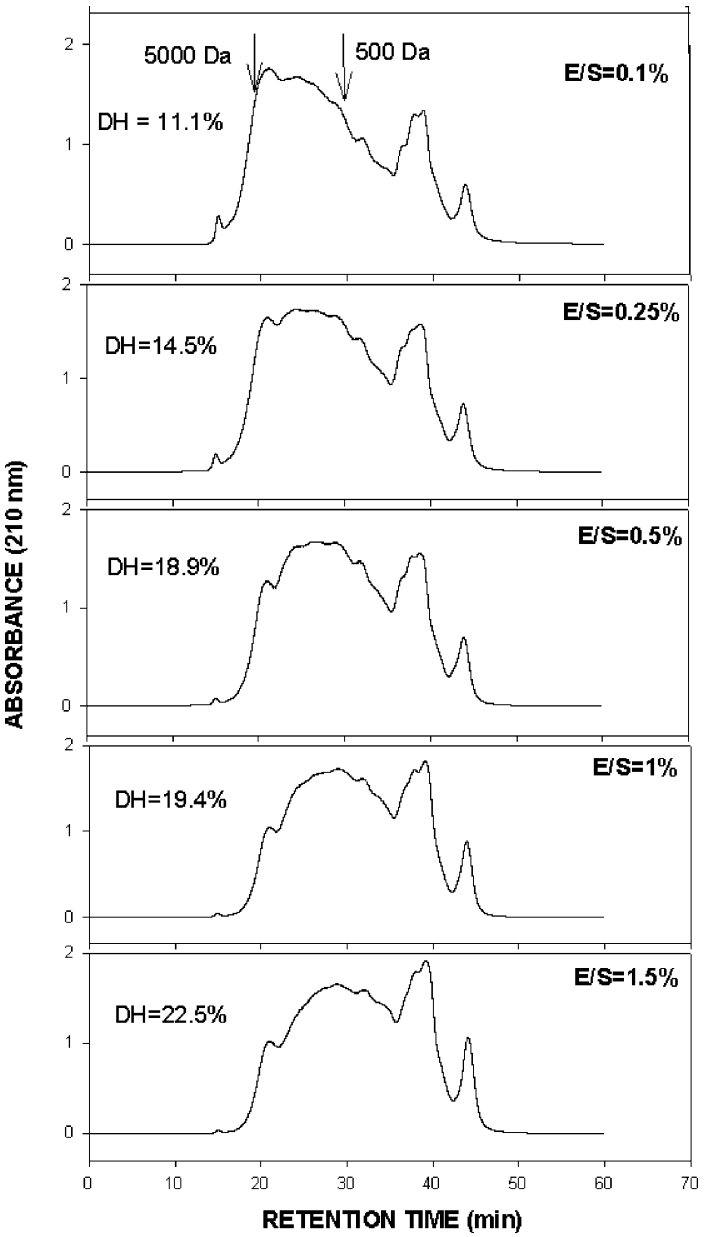

Fig. 3. Size exclusion chromatography of hydrolysates on SUPERDEX PEPTIDE HR 10/30 column. Samples were prepared using the enzyme "Umamizyme" at different *E*/*S* ratios (w/w protein) ranging from 0.1 to 1.5%. Hydrolysis was stopped after 4 h. The reaction was run at pH 7 and 45 °C.

Hydrolysates with a 11.1% DH was characterized by a low percentage of peptides with molecular weight above 5000. A regular decrease of this fraction was observed as the enzyme/substrate ratio increased (Table 1). The enzymatic treatment by Umamizyme of the tuna stomach proteins yielded a high quantity of small peptides below 500 Da ranging from 39% (11.1% DH) to 58% (22.5% DH).

Table 1
Molecular weight distribution (percent of total area) from size exclusion chromatography of hydrolysates on SUPERDEX PEPTIDE HR 10/30 column

	E/S ratio (w/w) (w/w protein) (%)				
	0.10	0.25	0.50	1.00	1.50
MW > 5000	13.1	8.6	5.5	3.7	3.6
2000 < MW < 5000	20.6	18.1	16.1	13.3	12.1
1000 < MW < 2000	14.8	14.3	14.7	13.8	12.6
500 < MW < 1000	12.8	13.7	14.7	14.7	13.9
MW<500	38.7	45.3	49	54.5	57.8
DH (%)	11.1	14.5	18.9	19.4	22.5

Samples were prepared using the enzyme "Umamizyme" at different *E/S* ratios (w/w protein) ranging from 0.1 to 1.5%. Hydrolysis was stopped after 4 h. The reaction was run at pH 7 and 45 °C.

3.3. Nitrogen recovery (NR)

When the enzyme/substrate ratio increased, the nitrogen recovery resulted in higher NR after a 4-h reaction up to a boundary value of 57% (Fig. 4). Similar NR values have been observed in hydrolytic reactions of fish proteins using fungal proteases [25,31]. A non-linear trend between enzyme concentration and NR can be seen. NR reflected the yield that can be recovered from the hydrolysis process, whereas, DH was used as an indicator for the cleavage of peptide bonds [14]. These results agreed with the findings of Diniz and Martin [25] who observed a quadratic trend between *E/S* ratio and NR. A sharp increase in NR occurred with *E/S* ratio ranging from 0.1 to 1%. However, no appreciably increase for NR was found with treatment of enzyme/substrate ratio above 1%.

3.4. Study of the limiting factors of hydrolysis

The asymptotic shape of the hydrolysis curves obtained using "Umamizyme" was comparable to those of Alcalase® 2, 4L-assisted reactions on food proteins. In fact, the proteolysis of casein, gelatine, maize and soy isolates and chickpea protein have been reported to present similar profiles [23,28]. The hydrolytic reaction depends on the availability of susceptible bonds, on which the primary enzymic attack is concentrated, and on the physical structure of the protein molecule [32]. We have previously shown that, during the solubilisation of tuna stomach using Alcalase® 2, 4L, the decrease in the concentration of peptide bonds available for hydrolysis due to the heating treatment of the substrate before hydrolysis was one of the main factor controlling the hydrolysis rate [8].

Very little is known about the Umamizyme preparation and about the factors limiting the enzyme activity. Therefore, the same investigation as we had

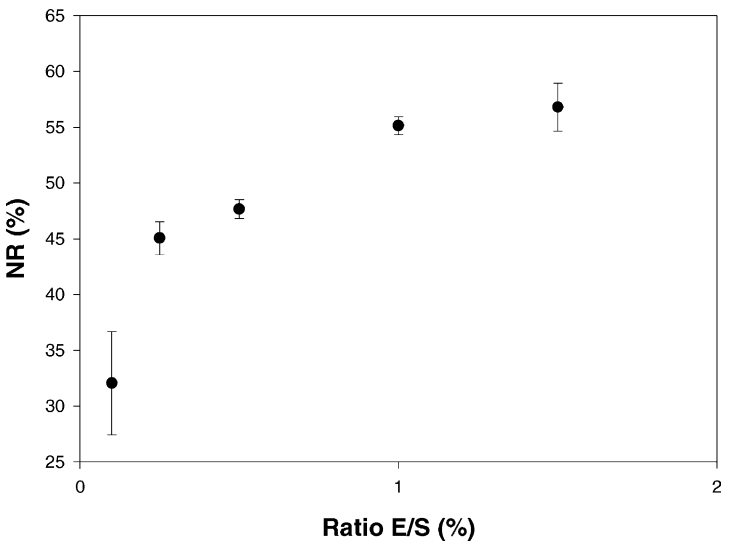

Fig. 4. Effect of the *E/S* ratio (w/w protein) on the nitrogen recovery (NR) of tuna stomach hydrolysates prepared using "Umamizyme". (DH; mean of three replicate samples ± S.D.).

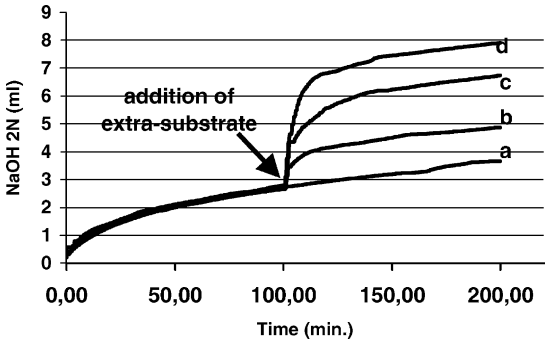

Fig. 5. Effect of extra-substrate addition during the hydrolysis. Addition of extra-substrate in an amount corresponding to 25% (b), 50% (c) and 75% (d) of the original mass substrate at 100 min. Reference (a): "Umamizyme" $E/S = 0.25\%$. Reaction conditions: pH 7, 45 °C.

conducted using Alcalase® 2, 4L was carried out with the Umamizyme preparation. The hypothesis explaining the downward curvature could be related to one of the following phenomena:

(a) a decrease in the concentration of peptide bonds available for hydrolysis;
(b) enzyme inhibition;
(c) enzyme deactivation.

In order to study the first possibility, an experiment was carried out during which, after 100 min of hydrolysis, an addition of extra-substrate was made in an amount corresponding to 25, 50 and 75% of the original mass of substrate. It can be seen from Fig. 5 that a sharp increase in the amount of alkali consumed to keep the pH constant during the reaction took place immediately after the extra-substrate addition. The final DH (%) was calculated at 200 min taking into account the amount of extra-substrate (M_p) added. The relative DH (%) was deduced from the final DH (%) (a value equal to 100 was attributed to the DH (%) obtained at 200 min without extra-substrate addition). The addition of extra-substrate induced a start up again of hydrolysis (calculated from the amount of alkali consumed) which was not directly proportional to the amount of extra-substrate added, whereas, the final degree of hydrolysis increased slightly, ranging from 14.74 to 17.08%. When this experiment was conducted using Alcalase® 2, 4L, we had shown that the final DH remained constant, whatever the amount of substrate added [8]. In the case of Umamizyme, a non-linear

Table 2
Effect of extra-substrate addition on the degree of hydrolysis evaluated after 200 min of hydrolysis

	Amount of extra-substrate added (%)			
	0	25	50	75
Amount of alkali consumed (ml)	3.7	4.9	6.7	7.9
Final DH(%) at 200 min	13.9	14.7	16.8	17.1
Relative DH (%)	100	106	121	123

relationship was established between the relative DH (%) and the substrate exhaustion suggesting that the decrease of the reaction rate was partly due to the decrease in the peptide bonds available for hydrolysis (Table 2).

To investigate the existence or non-existence of enzyme deactivation, an experiment was carried out with intermediate addition of enzyme (after 100 min of reaction, 100 200 and 400% of fresh enzyme was added). Fig. 6 showed the occurrence of a small increase in the hydrolysis rate as a result of this addition.

These results pointed out the part of enzyme in the decrease of the reaction rate. To obtain more information about enzyme deactivation or inhibition, the activity of Umamizyme was assayed during the course of hydrolysis according to the Nielsen and Kirkegaard procedure [26]. The diameter of disc-sharp opaque zones observed for holes containing Umamizyme with a ratio *E*/*S* of 0.25% (w/w protein) were compared at

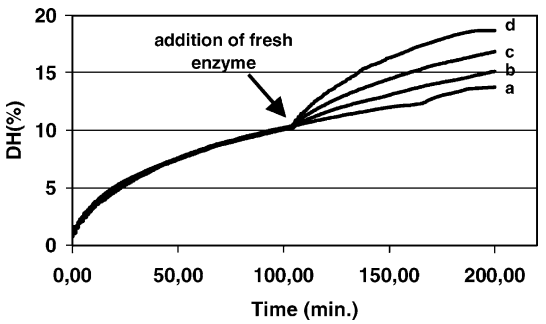

Fig. 6. Effect of fresh enzyme addition during the course of hydrolysis. Enzyme was added in an amount corresponding to 100% (b), 200% (c) and 400% (d) of the original enzyme concentration after 100 min. Reference (a): "Umamizyme" $E/S = 0.25\%$ (w/w protein). Reaction conditions: pH 7, 45 °C.

Table 3
Detection of residual proteolytic activity over the time for tuna hydrolysate treated with a ratio $E/S = 0.25\%$ (w/w protein)

	Time (min)						
	0	25	75	100	125	175	205
Decrease (%)	0	13.3	17	19.4	20.9	24.2	27.8
Area (%)	100	86.6	83	80.6	79.1	75.8	72.1

the beginning and during the course of the hydrolysis (Table 3). A regular decrease of the areas was observed during the course of hydrolysis suggesting an enzyme deactivation or an enzyme inhibition by the inhibitory peptides, which are continuously solubilised during hydrolysis. Such behaviour also suggested possible deactivation of the enzyme over the time due to a low stability at 45 °C for several hours, including the possibility that the enzyme hydrolyses itself. This was suggested by Diniz and Martin [19] using Alcalase® 2, 4L for the hydrolysis of dogfish proteins and is in close agreement with early findings on the hydrolysis of other kind of protein substrates. This observation together with the results of the extra enzyme and substrate additions makes it possible to conclude that the shape of the hydrolysis curves can be explained as a result of the lack of peptide bonds available for hydrolysis combined to a partial enzyme deactivation during the course of hydrolysis.

4. Conclusion

This study showed that the protease Umamizyme is suitable for use in the production of hydrolysate from tuna stomach proteins. The lowest stability of Umamizyme and the highest sensibility to deactivation or inhibition by the inhibitory peptides solubilised during hydrolysis compared to Alcalase® 2, 4L is not a major disadvantage because the most important factor is not an extended process but a protein degradation sufficient for the production of peptides of various size. Some of them are likely to retain biological properties which could be of specific interest for aquaculture. Molecules possessing structural and/or functional characteristics of gastrin and cholecystokinins (secretagogue molecules exhibiting a large spectrum of activities ranging from the stimulation of protein synthesis to the secretion of digestive enzymes) or cellular growth factors have already been detected in tuna stomach hydrolysates prepared using Alcalase® 2, 4L [4]. As the controlled hydrolysis of tuna stomach proteins through the action of Umamizyme provided a high proportion of small soluble peptides below 5000 Da, the biological effect of the hydrolysates obtained needs to be thoroughly investigated.

Acknowledgements

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