Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacares*) wastes using Alcalase

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Abstract

Enzymatic hydrolysis of tuna stomach proteins by Alcalase was investigated in a batch reactor. The influence of the process variables (enzyme/substrate ratio; effect of intermediate substrate and enzyme addition) was studied with regards to the extent of proteolytic degradation and to the molecular weight distribution of the peptides. A linear correlation was found between the degree of hydrolysis (DH) and the enzyme concentration. After addition of extra substrate during the course of hydrolysis, the final DH obtained was proportional to the substrate added, suggesting that the concentration of hydrolysable bonds was one of the main factors controlling the hydrolysis rate. Preliminary results showed that tuna protein hydrolysates performed effectively as nitrogenous source in microbial growth media. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The use of fish wastes has been of increasing interest over the past 10 years as biomass from marine origin is considered as a safe material and provides proteins with high nutritional properties and a good pattern of essential amino acids [1–4]. The tuna wastes (Thunnini) constitute a biomass of particular interest to upgrade because of the global economic importance of tunas and their international trade for canning. The global production of the principal tuna market species has tended to increase continuously, from below 0.5 million tons in the early 1950s to 3.1 million tons in 1994. Yellowfin is commercially the second most important species of tuna and, in 1994, accounted for about 1.1 million tons or 35% of the total catches [5]. Since only the white meat of tuna is used in canning, a sizeable amount of solid wastes including viscera, head, skin, bone and some muscle tissue is generated and can be as high as 70% of the original material.

Traditionally, fish wastes have been used as fishmeal for animal feeding [2]. Another way of upgrading for fish proteins has been the production of Fish Protein Hydrolysates (FPH) in controlled conditions [3,6,7]. Protein solubilisation from raw fish materials has been carried out mostly by biological methods, especially by enzymatic hydrolysis. Autolytic process, which depends only on the action of the natural digestive enzymes occurring in the fish itself, is considered to be economically interesting [8]. However, the use of exogenous commercial enzyme is

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preferred in autolysis by endogenous enzymes since the hydrolysis and the properties of resulting products (i.e. the peptide chain length of the hydrolysates) can be controlled [1,2]. A lot of commercial proteases from plant, animal or microbial sources have been reported as the potential proteinases for hydrolysing fish proteins [3,7,9–12]. From a technical and economic point of view, enzymes from microbial sources operating at alkaline pH such as Alcalase were shown to be one of the most efficient in the hydrolysis of fish proteins [13].

There is a recent interest for finding new applications — with better added value — to the FPH. In our laboratory, the European research project FAIR CT 97-3097 explores the possibility of obtaining biologically active peptides from hydrolysates of marine food processing wastes. As an example, cod hydrolysates prepared from heads, stomach and viscera gave positive results in the calcitonin gene-related peptide (CGRP) radioimmunoassay [14]. The use of FPH as nitrogenous substrates for growth stimulation of microorganisms is also investigated since growth substrate costs often make up the major part of the production cost of microbial cells and bioproducts from the fermentation industry. Fish peptones need to be thoroughly investigated to find industrial applications.

The purpose of this work is to study the effect of Alcalase on the hydrolysis of proteins from tuna wastes and to explore the possibility of obtaining hydrolysates of controlled molecular weight peptides that support the growth of microorganisms in culture.

2. Materials and methods

2.1. Materials and reagents

Yellowfin tunas (*Thunnus albacares*) caught in the Indian Ocean and immediately frozen once on board were obtained from a fish processing company «Paulet» (Douarnenez, France). The stomachs were taken from frozen fish and washed with water. Heat inactivation of endogenous stomach enzymes (100°C — 20 min) was carried out prior to pH adjustment and addition of Alcalase 2.4 L.

Alcalase 2.4 L (a declared activity of 2.4 AU/kg and a density of 1.18 g/ml) was provided by Novo Nordisk (Denmark). All reagents used were of analytical grade.

2.2. Preparation of the hydrolysate

Hydrolysis experiments were carried out in a 1-l reactor using the pH-stat method in controlled hydrolysis conditions (pH, temperature, enzyme concentration and stirring speed). Enzyme concentrations varied in the different tests covering the range of 5.6–85 AU/kg of wet stomach, i.e. Alcalase was added to the sample at enzyme/substrate (E/S) concentration ranging from 0.2% to 3% (wet weight basis). All experiments were carried out in duplicate. During each hydrolysis, pH was maintained constant at the desired value by addition of 2 N NaOH. Reactions were terminated by heating the solution to 95°C for 20 min, which assured the inactivation of the enzyme. The resulting slurry was centrifuged at $20\,000 \times g$ for 20 min.

Many authors [6,9,15] reported successful hydrolysis of plant or animal proteins with Alcalase at pH 8.0 and 50°C, so proteolysis was conducted using these pH and temperature, also following the indications of maximum activity and stability of the Alcalase provided by Novo Industry [16]. Flowsheet for the hydrolysate production is reported in Fig. 1.

2.3. Determination of the degree of hydrolysis (DH)

Reactions were monitored by measuring the extent of proteolytic degradation by means of the DH according to the pH-stat method described by Adler-Nissen [17]. The DH is defined as follows:

$$DH(\%) = \frac{\text{Number of peptide bonds cleaved}}{\text{Total number of peptide bonds}} \times 100.$$

The values for DH can be determined using the following equation:

$$DH(\%) = \frac{BN_b}{M_p \alpha h_{tot}} \times 100,$$

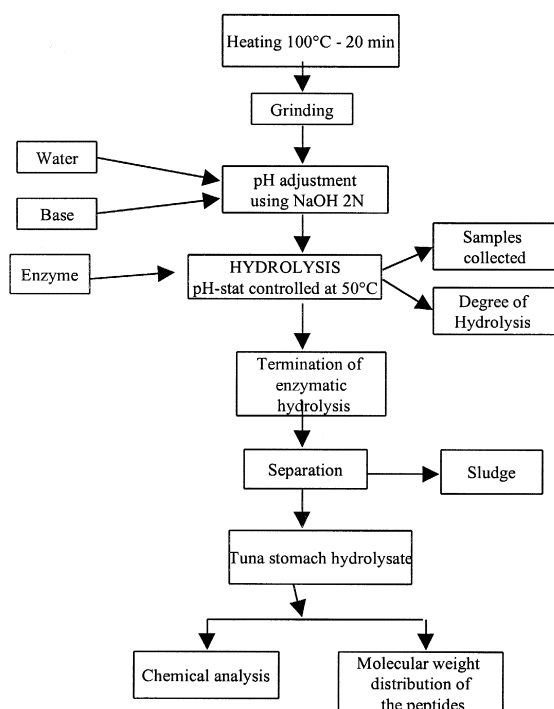where DH is the percent ratio between the number of peptide bonds cleaved (h) and the total number of

Fig. 1. Flowsheet for the production of protein hydrolysate from tuna stomach.

2.4. Size exclusion chromatography (SEC)

The molecular weight distribution of peptides for each sample was analysed using FPLC gel filtration. The liquid chromatographic system consisted of a Waters 600 automated gradient controller pump and a Waters 996 photodiode array detector. The SEC column was a Superdex Peptide HR 10/30 column from Pharmacia (fractionation range of the column was 7000 to 100 Da). The mobile phase (isocratic elution) consisted of water with TFA 0.1% and acetonitrile (70:30). The flow rate was 0.5 ml/min. MILLENIUM software was used to collect, plot and process the chromatographic data.

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 has been established. Samples injected were dissolved in mobile phase and filtered at 0.2 μm before injection. Absorbance was monitored at 220 nm. For each chromatogram, peptides were sorted out into three fractions from 0 to 500 Da (fraction III), 500 to 2000 Da (fraction II) and above 2000 Da (fraction I). The relative areas of each fraction were given in percentage of the total area.

2.5. Microbial cultivations

Four fish peptones were compared to a reference peptone from casein (Table 1).

2.5.1. Microorganisms and cultivation media

The bacteria (*Escherichia coli* ATCC 25922, *Lactobacillus casei* ATCC 7469), the yeasts (*Sporobolomyces odorus* CBS 2636, *Saccharomyces*

peptide bonds in the substrate studied (h_{tot}). The variable B is the amount of alcali consumed to keep the pH constant during the reaction, N_{b} is the normality of the alkali, M_{p} is the mass of the substrate (protein, determined as $N \times 6.25$) in the reaction and α is the average degree of dissociation of α-NH$_2$ groups released during hydrolysis.

Table 1
Short presentation of the peptones used in this study

Trade name	Raw material	Manufacturer	Presentation (aspect)	Dry matter (g DM/100 g of product)	Nitrogen content (g N/100 g of dry matter)[a]
Bacto Tryptone	casein	Difco (USA)	powder	95.06	11.5
–	cod viscera	Biotec Maczymal (N)	powder	89.90	9.5
–	tuna stomach	Lumaq (F)	powder	94.84	13.4
Marine peptone S490	salmon co-products	Primex (N)	liquid	66.53	10.5
Fish peptone No. 1	various fish	Difco (USA)	powder	94.53	10.6

[a] Kjeldahl method.

cerevisiae from IUT Biologie Appliquée, Quimper), and the fungi (*Aspergillus niger* from ESMISAB, Brest, *Penicillium roqueforti* CSL-PV) were grown at 25°C in liquid media previously autoclaved at 121°C for 15 min. The medium consisted of (w/w): 1.5% glucose, 0.5% peptone (except salmon peptone in liquid form, 0.5% v/v), 0.2% KH_2PO_4, 0.013% $CaCl_2$, 2 H_2O, 0.001% $FeSO_4$, 7 H_2O, 0.3% $MgSO_4$, 7 H_2O, pH 6.0. Cultivation was performed for 3 to 5 days on a rotatory shaker (150 rpm) in 250-ml culture flasks containing 100 ml of medium.

2.5.2. Growth kinetics, modeling the growth curve

Bacterial and yeast growths were followed using optical density measurements (650 nm). Each growth curve for a microorganism/peptone combination was obtained from four cultures.

A lot of mathematical models can be used to obtain lag phase (λ), maximum growth rate (μ_{max}) and maximum biomass at the stationary phase (A) [18]. The GOMPERTZ model (Eq. 1), well-suited for such a purpose, was applied to the growth curves obtained on peptones.

$$\log \frac{N}{N_0} = A \exp\left(-\exp\left(\frac{\mu_{max} \exp(1)(\lambda - t)}{A} + 1\right)\right), \qquad (1)$$

where N_0 = initial population; N = population at instant t.

Calculations were made using EXCEL (Microsoft) using the least square method to adjust the model to

Fig. 3. Relation between $\log_{10}$ (enzyme concentration) and DH for tuna stomachs treated with Alcalase. The hydrolytic reaction was run for 5.5 h at pH 8. Length of hydrolysis period (h): (a) 0.3; (b) 0.8; (c) 1.4; (d) 2.2; (e) 5.5.

the data, and correlation coefficient to estimate the fitness between the data and the model.

3. Results

3.1. Effect of enzyme concentration on the DH

Fig. 2 shows the hydrolysis curves obtained for different initial enzyme concentrations. The DH in-
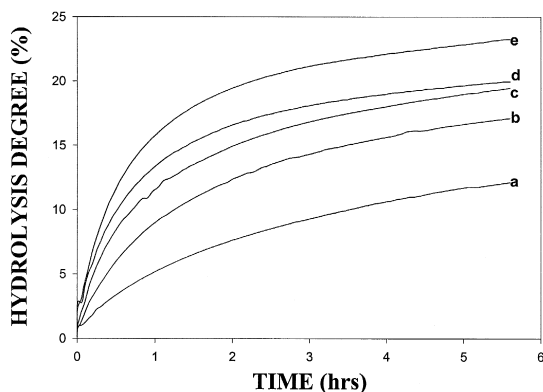

Fig. 2. Hydrolytic curves for tuna stomach treated with Alcalase. Enzyme concentrations (AU/kg). (a) 5.6; (b) 14.4; (c) 28.3; (d) 45.3; (e) 85. Reaction conditions: pH = 8; temperature = 50°C.
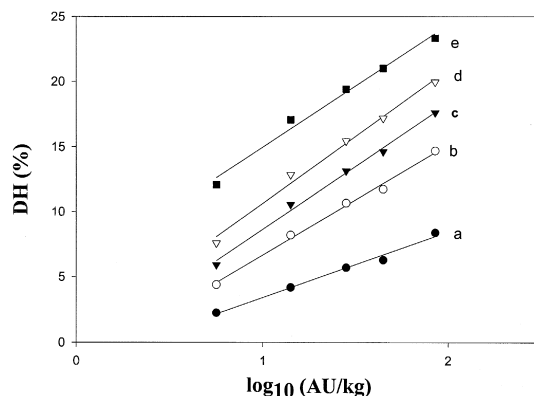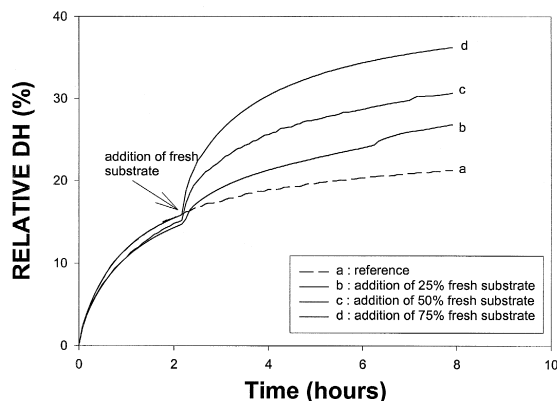
Effect of addition of fresh substrate

Fig. 4. Effect of extra-substrate addition during hydrolysis. Extra-substrate was added in an amount coresponding to 25%, 50% and 75% of the original mass of substrate at $t = 2.2$ h. Reaction conditions: pH = 8; temperature = 50°C.

Table 2
Effect of extra-substrate addition on the final and relative DH

Addition of extra-substrate (%)	0	25	50	75
Final DH (%)	21.12	21.4	20.5	20.7
Relative DH (%)	100	126	145	171

creased with increasing enzyme concentration. A DH up to 23% was observed with the highest enzyme concentration. Prolonging the reaction beyond 5.5 h did not produce any significant improvement in the DH. Significant changes in DH occurred with the enzyme treatment at concentrations ranging from 0 to 28.3 AU/kg. Less significant increases were found with treatment enzyme at concentration above 28.3 AU/kg. Similar curves were reported for the enzymatic hydrolysis of sardine [12], capelin [3], and shark muscle [9].

When log10 (enzyme concentration) versus DH (%) was plotted, a linear relationship was observed (Fig. 3). The correlation coefficients were obtained for Alcalase at different enzyme concentrations ($R^2 = 0.99$). From this relationship, the exact concentration of enzymes required to hydrolyse tuna proteins to a required DH, from 0.5 to 5.5 h, could be calculated.

3.2. Study of the reaction mechanism

The mechanism of hydrolysis of tuna proteins was relatively complex to analyse because the substrate consisted largely of insoluble proteins, whereas the enzymes were soluble. Möhr [8] suggested that, in such a case, the overall reaction involved at least two steps. In the first step, the enzyme molecules become associated with and bound to the fish particles. Subsequently, hydrolysis took place, resulting in the release of soluble peptides and amino acids. After addition of the enzyme, there was an initial rapid phase during which a large number of peptide link-
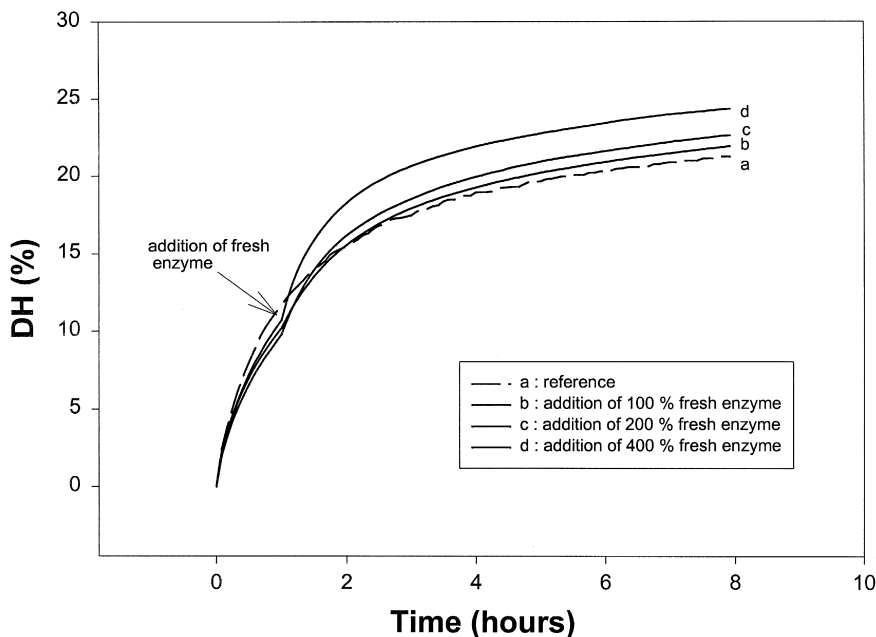
Effect of addition of fresh enzyme

Fig. 5. Effect of fresh enzyme addition during hydrolysis. Fresh enzyme was added in an amount corresponding to 100%, 200% and 400% of the original enzyme concentration after 1 h. Reaction conditions: pH = 8; temperature = 50°C.

ages were cleaved per unit time, and during which a large proportion of soluble material was released into solution. The most compacted core proteins were hydrolysed more slowly [2]. Consequently, the rate of enzyme cleavage of peptide bonds controlled the overall rate of hydrolysis. At the same time, available substrate decreased as time reaction increased.

Fig. 2 shows the hydrolytic curves obtained with Alcalase at different enzyme concentrations. It appeared that, except for the lower enzyme concentration (5.6 AU/kg), the hydrolytic curves had the same characteristics. Each hydrolysis profile was characterised by a high initial rate for the first 1.5 h, followed by a rapid decrease in rate tending towards a boundary value. Moreno and Cuadrado [15], studying the enzymatic hydrolysis of chick pea proteins by Alcalase at 50°C and pH 8, suggested that the hydrolysis curve downward tendency could be attributed to one of the following phenomena:

(a) a decrease in the concentration of peptide bonds available for hydrolysis;
(b) an enzyme activity decrease; and
(c) a product inhibition.

So, in order to study the first possibility, an experiment was carried out during which, after 1.7 h of hydrolysis, and after nearly reaching the straightest part of the curve for an enzyme concentration of 14.16 AU/kg, an addition of extra substrate was made in an amount corresponding to 25%, 50% and 75% of the original mass of substrate. Relative DH was calculated, taking into account only the amount of original substrate in the reaction (M_p), whereas final DH was obtained taking into account the original amount of substrate plus the amount of substrate (ranging from 25% to 75%) added during the course of hydrolysis.

It can be seen from Fig. 4 that a sharp increase in the amount of alcali consumed to keep the pH constant during the reaction took place, i.e. the relative DH obtained was proportional to the substrate added (Table 2). On the contrary, final DH remained constant (from 20.7% to 21.2 %, whatever the amount of substrate added).

To investigate the possibility of a decrease in enzyme activity, an experiment was carried out with intermediate addition of enzyme (after 1.7 h of reac-

tion, 100%, 200% and 400% of fresh enzyme were added). Fig. 5 indicates that no notable change was observed in the trend.

3.2.1. Effect of addition of fresh enzyme during the hydrolysis

The shape of hydrolysis curves could also be explained as a result of the action of inhibitory peptides, which were continuously solubilised during the hydrolysis. Moreno and Cuadrado [15] pointed out the presence, in chick pea hydrolysates, of an inhibitor of subtilisin, the main component of Alcalase. Our preliminary results showed that for the cooked tuna protein hydrolysis, the concentration of hydrolysable bonds was the main factor controlling the hydrolysis rate and could be explained by the heat treatment of the starting material before hydrolysis.
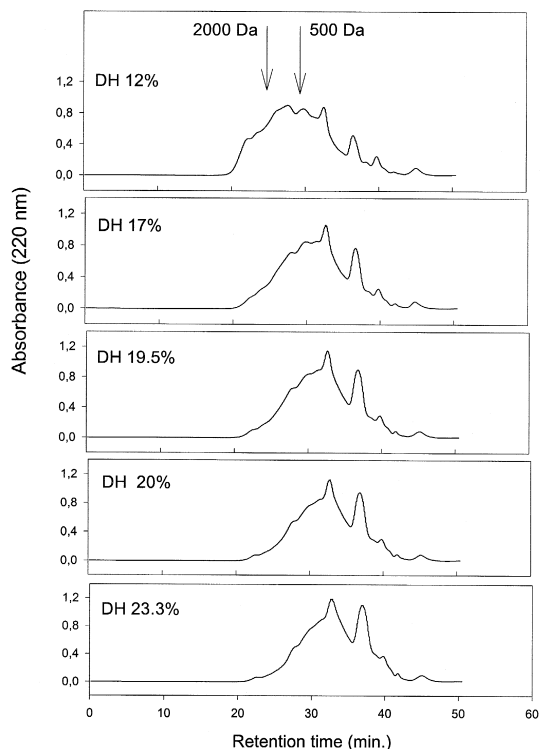

Fig. 6. Elution of tuna stomach hydrolysate from Superdex HR 10/30. Samples were prepared using the enzyme Alcalase at different concentrations ranging from 5.6 to 85 AU/kg at pH 8 and 50°C. Hydrolysis was stopped after 5.5 h.

As a matter of fact, one of the problems usually encountered with the protein hydrolysates from fish viscera was the lack of reproducibility caused by the presence of endogenous proteases, which could act on the hydrolysis process. Concerned with this, tuna stomachs were cooked before enzymatic treatment in order to inactivate the endogenous proteases, mainly pepsine. Consequently, protein denaturation was a reasonable explanation for lost ability of Alcalase to hydrolyse efficiently the heated proteins because of a lower protein flexibility. Nevertheless, in the case of the product we intended to develop, i.e. FPH as potential source of bioactive peptides or as nitrogen substrate for fermentation media, it was essential to control the size of the peptides obtained. This was accomplished with standardised initial material, thus free of by-side enzymatic activities, and the use of exogenous proteases for hydrolysis.

3.3. Study of chromatographic profiles

The tuna stomach hydrolysates were analysed using size exclusion chromatography (Figs. 6 and 7).

Samples were collected after 5.5 h of hydrolysis. The enzymatic treatment by Alcalase of the tuna stomach proteins produced major fractions with molecular weights ranging from 6500 Da to free amino acids. A decrease of the high molecular weight fractions was noticed as the enzyme/substrate ratio increased.

The results obtained showed that this technique was useful for comparing peptidic profiles from different runs and for checking the profile adequacy of identical runs (unpublished results). Zwietering et al. [19] outlined the limits of the SEC, i.e. (i) the approach used could result in underestimating small peptides and free amino acids and (ii) it could not be applied to absolute determination of molecular weight distribution. However, the SEC technique was a very valuable tool suitable for the follow up of proteolysis and for the routine analysis of a large number of samples.

3.4. Microbial growth

Tuna peptone produced in our laboratory was included in the culture media of six microorganisms
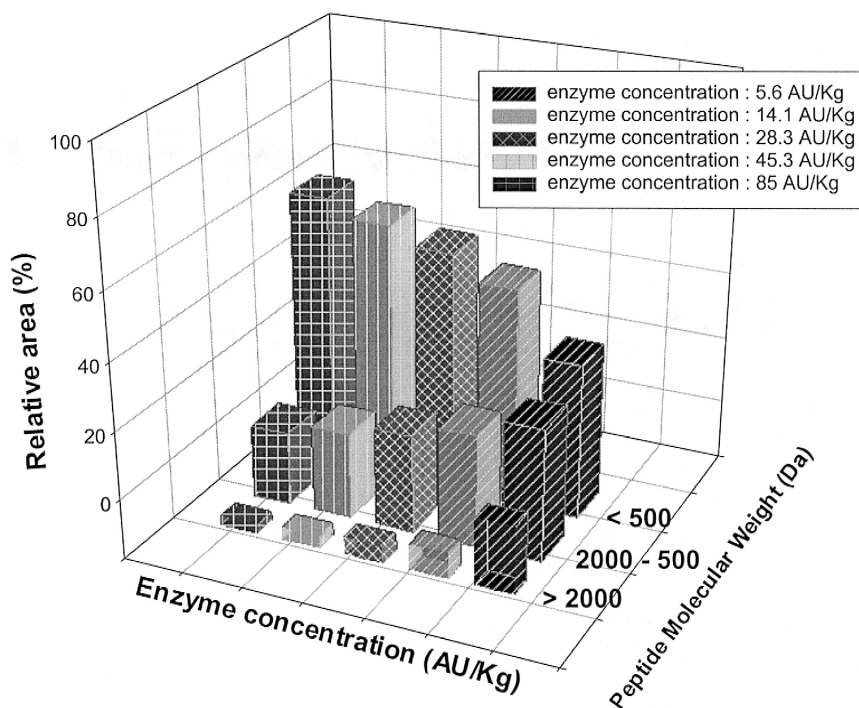

Fig. 7. Molecular weight distribution of peptides expressed in % of total area. Hydrolysates were prepared using Alcalase at concentrations ranging from 5.6 to 85 AU/kg and stopped after 5.5 h. Reaction conditions: pH = 8; temperature = 50°C.

belonging to bacteria, yeasts and fungi genera. Among bacteria, one gram negative was chosen, i.e. *E. coli*, as transformed *E. coli* is frequently used in biotechnology; this microorganism is rather easy to grow. The gram positive bacteria *L. casei*, which is harder to grow, is present in dairy starters, and is also used for lactic acid production or post-koji making. Among the yeasts, one Ascomycete, *Sac. cerevisiae* and one Basidiomycete, *Spo. odorus* were tested. *Sac. cerevisiae* is a very common yeast in biotechnology, in food manufacture (bakery, beer, wine making...) and *Spo. odorus* is used for aroma production. For the fungi, fish peptones were tested on *P. roqueforti*, which is used for cheese making (Roquefort) or aroma production (methyl ketones),

and on *A. niger*, which produces citric acid on an industrial scale.

Growth followed by spectrophotometric measurements allowed us to calculate lag phases, growth rates and maximum biomass at the stationary phase. Results obtained for the yeast *Spo. odorus* are given in Fig. 8 as an example.

Data from three fish peptones (including tuna) out of four tested were very close, so tuna peptone could compare with well-established industrial products like casein hydrolysate. As results obtained for the other five microorganisms were as good as for *Spo. odorus* (data not shown) [20], fish peptones — and the one tuna in particular — should have a promising future in biotechnology.
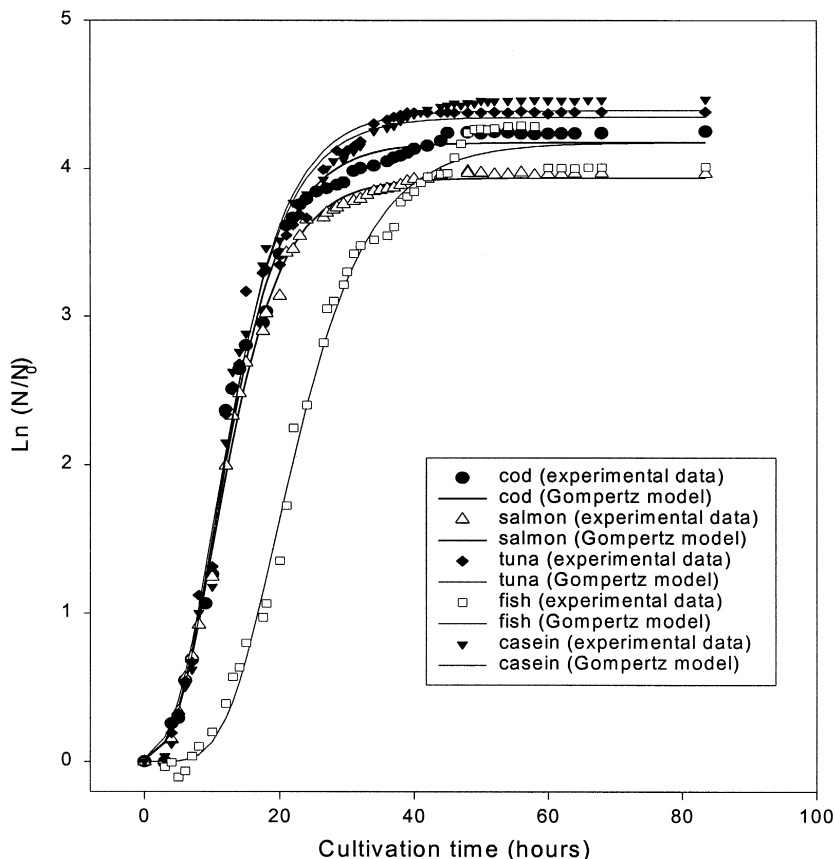

Fig. 8. The yeast *Spo. odorus* was grown at 25°C in a liquid medium that consisted of glucose (15 g/l), peptone (5g/l) (except salmon peptone in liquid form, 0.5% v/v) and mineral mix (0.2% KH_2PO_4, 0.013% $CaCl_2$, $2H_2O$, 0.001% $FeSO_4$, $7H_2O$, 0.3% $MgSO_4$, $7H_2O$), pH 6. A tuna hydrolysate was compared to three commercial fish peptones and to a reference peptone from casein (Bactotryptone DIFCO). Optical density (650 nm) was monitored during the growth. The GOMPERTZ model was applied to the growth curves obtained on peptones.

4. Conclusion

The controlled hydrolysis of tuna stomach protein through the action of Alcalase 2.4 L provided a high proportion of peptides from 6500 Da to di-peptides and free amino acids. The concentration of available hydrolysable bonds was one of the main factors controlling the hydrolysis rate. Freeze-dried hydrolysates were tested successfully as nitrogen substrate for microbial cultures. Further work will focus on the effect of the hydrolysis degree on the performance of peptones in microbial studies.

Acknowledgements

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