Food Chemistry 112 (2009) 604–608

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Advantages of a proteolytic extract by *Aspergillus oryzae* from fish flour over a commercial proteolytic preparation

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ARTICLE INFO

Article history:
Received 9 November 2007
Received in revised form 10 June 2008
Accepted 11 June 2008

Keywords:
Proteases
Aspergillus oryzae 2095
Solid–state fermentation
Fish flour
Degree of hydrolysis

ABSTRACT

Proteolytic extract (CPE) was produced by *Aspergillus oryzae* 2095 under solid state fermentation using a mix of fish flour with polyurethane foam (70:30, w/w) of particle size of 0.5 mm. The proteolytic activity from CPE was compared to a commercial protease (Flavourzyme 500 MG®). The maximal activity for both extracts was found at pH 8 and 50 °C. The half-lives of CPE and Flavourzyme 500 MG® were 52 and 25 min at 50 °C, respectively. Furthermore, the hydrolytic activity for both extracts was tested on muscle of giant sea bass (*Epinephelus morio*), CPE produced a higher degree of hydrolysis (DH) than Flavourzyme 500 MG® (22.2 and 9.1%, respectively) under optimal experimental conditions for each extract.

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1. Introduction

Total sale of proteases account for about 60 percent of the enzyme world market and have numerous commercial applications in the food industry (Germano, Pandey, Osaku, Rocha, & Soccol, 2003), for protein-degrading additives in detergents, leather dehairing and bating, silver recovery, cleansing solutions, processing of proteinaceous waste, peptide synthesis, and alike (Bakhtiar, Andersson, Gessesse, Mattiasson, & Hatti-Kaul, 2002). Forty percent of the enzymes used today are produced by microorganisms, from them the use of fungi strains for enzyme production have a myriad of advantages as they are generally recognized as safe (GRAS) (Sandhya, Sumantha, Szakacs, & Pandey, 2005) and produce a wide range of extracellular enzymes, therefore the downstream processing is more simple compared to the enzymes obtained from plants or animals (Gupta, Beg, Khan, & Chauhan, 2002a). Additionally, *Aspergillus oryzae* is used extensively to produce a wide variety of industrial enzymes such proteases which have been produced either by solid–state (SSF) or by submerged fermentation (SmF) (Sandhya et al., 2005). Advantages of SSF include simplicity, lower production costs, low wastewater output and high enzyme yields. The most common substrates for SSF include agroindustrial residues (Mitchell, Berovic, & Krieger, 2000) and other disposal materials such as fish processing by-products that could otherwise represent pollution and disposal problems (Ellouz, Bayoudh, Kammoun, Gharsallah, & Nasri, 2001).

Some reports have shown that proteases production by bacteria using fish processing by-products like sardinelle heads and viscera flour (Ellouz et al., 2001), under-utilized fish such as *Johnius dissumeri* (Venugopal, 1994) and shrimp and crab shell powder as substrate (Wang, Law, & Webb, 2005a). Proteases have a wide industrial application (Banerjee, Sani, Azmi, & Soni, 1999; Germano et al., 2003; Kumar & Takagi, 1999; Rao, Tanksale, Ghatge, & Deshpande, 1998; Wang et al., 2005a). Recently, proteases are being used to produce fish protein hydrolysates (FPH), which allow the fish by-products recovery giving to the peptides with biomedical, nutraceutical, antioxinantive, and functional properties including improved solubility, emulsifying, foaming and water-binding (Kim et al., 2001; Kristinsson & Rasco, 2000a).

The aim of this study was to produce a proteolytic extract by *A. oryzae* 2095 under SSF using fish flour as a substrate. The proteolytic extract was characterized in terms pH and temperature for optimal activity and stability and degree of hydrolysis (DH) on fish muscle, values obtained were compared with a commercial extract (Flavourzyme 500 MG®) under optimal activity conditions for each extract.

2. Experimental

2.1. Microorganism and inoculum preparation

A. oryzae 2095 from STCC (Spanish Type Culture Collection, Valencia, Spain) was grown on potato–dextrose agar (BIOXON, Mexico City, MX) plates. Conidiospores were harvested from a 7-days-old culture by suspending in sterile water containing 0.1%

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0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.06.016

Tween 80. Spore suspension was adjusted to desired concentration after counting in a Neubauer chamber (Brand, Germany).

2.2. Culture medium

Fish flour (FF) was purchased from a local feed animal shop (Protein content 0.63 protein g/FF g). It was sieved to obtain an homogeneous size particle of 0.84 mm, then mixed with polyurethane foam (PUF) (Expomex, Edo. Mex., MX) which was previously washed (Zhu, Knol, Smits, & Bol, 1996) and ground to reach particle size of 0.5 mm. FF and PUF were dried in an oven for 12 h at 80 °C. FF was mixed with PUF in a 70:30 (w/w) ratio. The powder mixture was sterilized at 121 °C for 15 min. The cultures were moistened with sterile 0.2 M phosphate buffer pH 7 to the desired water content (50%) and then used as the SSF culture medium.

2.3. Fermentation

Solid fermentation was carried out in packed tubular reactors (2.5 cm diameter for 22 cm length (Saucedo-Castañeda et al., 1994) with 30 g of culture medium as described above, previously inoculated with 4.5×10^7 spores/g of protein and incubated (72 h) under static conditions at 30 °C with an air flux of 40 cm^3 per min. Samples were taken at regular intervals (0, 24, 36, 40, 44, 48, 60, and 72 h), pH and proteolytic activity progress were determined.

2.4. Proteolytic extract

Crude proteolytic extract (CPE) was obtained by adding 30 ml of 0.2 M phosphate buffer (pH 7) and vigorously mixed by using a vortex for 1 min. The solid phase was separated by centrifugation (Allegra 25R Centrifuge, Beckman Coulter, CA, USA) for 15 min at 21,000g at 4 °C and then filtered under vacuum through a Whatman filter paper No. 40. The filtrate was used as a CPE.

2.5. Proteolytic activity assay

Proteolytic activity for CPE and Flavourzyme 500 MG® (endoproteinase and exopeptidase from *A. oryzae* from Novozymes, (Bagsvaerd, Denmark) was determined as follows: 2% casein (Hammerstein, Boehringer Mannheim Corp., Indianapolis, IN, USA) prepared in 0.2 M buffer at desirable pH (sodium phosphate for pH 6 and 7; Tris–HCl for pH 8 and 9; and carbonate–bicarbonate for pH 10). A mixture of 0.25 ml of casein solution and 0.25 ml enzyme extract (1:5 diluted for CPE and 1:100 for Flavourzyme 500 MG® with the appropriate buffer) was incubated at 30 °C for 10 min. The reaction was terminated by adding 0.5 ml of 0.4 M trichloroacetic acid (J.T. Baker, Phillipsburg, PA, USA); this mixture was then centrifuged at 11,100g for 15 min at 4 °C. The supernatant (0.25 ml) was added to 1.25 ml 0.4 M sodium bicarbonate (J.T. Baker) followed by 0.25 ml of 0.4 M of Folin–Ciocalteu Phenol Reagent (1:5 diluted in water) (SIGMA, St. Louis, MO, USA). This reaction mixture was allowed to stand for 30 min at 30 °C. Absorbance was read against a blank at 660 nm. Samples were analyzed in triplicates. One unit of protease (U) was defined as the amount of enzyme which yields the colour equivalent to 1 μM of tyrosine in 1 ml reaction per minute at 30 °C at each pH value assayed.

2.6. Effect of temperature and pH on enzyme activity

The optimum temperature and pH for CPE and Flavourzyme 500 MG® was determined using casein as substrate as described above. Temperature and pH varied from 30 to 70 °C and 6 to 10, respectively. Determinations were carried out in triplicate and data were shown as mean values.

2.7. Temperature and pH stability

To test thermal stability both extracts were incubated without substrate at temperatures ranging from 30 to 70 °C. The pH stability was measured in both diluted extracts (1:10, v/v) in the appropriated buffer (pH 6–10); samples were incubated without substrate at 4 °C. In both experimental units (0.25 ml) were withdrawn at regular intervals during 120 min. Residual activity was assayed at optimum conditions for each extract. Determinations were carried out in triplicate and data were shown as mean values.

2.8. Protein determination

The protein concentration of the CPE and Flavourzyme 500 MG® was determined according to Bradford (1976), BSA (Sigma®) was used as standard.

2.9. Enzymatic hydrolysis

Forty grams of giant sea bass (*Epinephelus morio*) (Supplied from Puerto Progreso, Yucatan to fish market "La Nueva Viga" in Mexico City) were homogenized in 100 ml of 0.2 M buffer Tris–HCl (pH 8) and filtered through a sieve. This preparation was use as fish muscle hydrolysable substrate (FMHS) at 1% protein concentration. Temperature and pH values of the FMHS were adjusted to pH and temperature optimal values before addition of the enzymatic extracts (CPE and Flavourzyme 500 MG® at a time). The pH-stat method was used to determine the DH of each enzymatic extract (Adler-Nissen, 1982) in a 250 ml vessel provided with automatics temperature, pH and stirring speed controls (Mettler DL 21 titrator, Hightstown, NJ, USA). Both proteolytic extracts were then added (at a time) to the FMHS mix at 24 U/g of substrate. The pH was kept constant by automatic addition of 0.5 N NaOH during hydrolysis. The amount added of NaOH was recorded at 1 min intervals for 1 h. A blank with out enzyme was carried out at same conditions, then NaOH consumed was subtracted from the amount the NaOH consumed by samples in order to eliminate auto-hydrolysis effects. Degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved and it was calculated according to earlier reports (Adler-Nissen, 1982).

3. Results and discussion

3.1. Culture conditions for protease production

Proteases production by *A. oryzae* 2095 under SSF is shown in Fig. 1. Enzyme production started after 24 h of culture, although

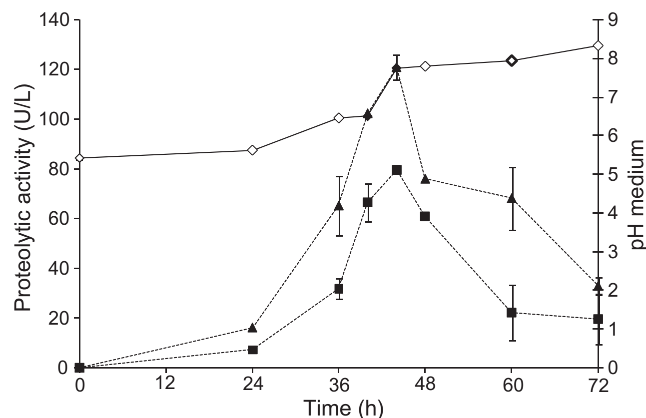

Fig. 1. Protease production by *Aspergillus oryzae* 2095 under SSF: (■) Neutral protease (pH 7), (▲) Alkaline protease (pH10), (◇) pH progress during fermentation.

maximum production was reached at 44 h for both neutral (pH 7) and alkaline (pH 10) proteases (79.71 Ul^{-1} and 120.78 Ul^{-1}, respectively). Time of maximal protease production was shorter compared to reports where agro-industrial residues were used. Maximum protease production by *A. oryzae* NRRL 1808 in SSF was showed at 72 h (Sandhya et al., 2005), while *A. oryzae* NRRL 2160 in SSF achieved maximal enzyme yields at 72–96 h (Battaglino, Huergo, Pilosof, & Bartholomai, 1991). A major peak of activity reached at 50 h by *A. oryzae* during batch fermentation was also reported (Wang et al. 2005a). Furthermore, decrease in protease production after 44 h could be due to nutrient limitations, pH variation (Chu, Lee, & Li, 1992; Sandhya et al., 2005), and production of amino acids and low-molecular-weight compounds which may inhibit protease activity (Gupta, Beg, & Lorenz, 2002b; Venugopal, 1994). Therefore, a reduction time in protease production was achieved by *A. oryzae* 2095 when compared with similar works.

Increase in pH medium was observed throughout fermentation (from 6 to 8); this could be related to ammonia production in the culture medium as a result of protein metabolism after proteases action. The major mechanism is likely to be the oxidation of amino acids during their utilization as energy source: $RCH(NH_3^+)COO^- + NO_2 \rightarrow NCO_2 + H_2O + NH_4^+ + OH^-$, in accordance to reports during tempe proteolysis (Sparringa & Owens, 1999), which is a medium with high protein content, similar to the fish flour used in this study.

3.2. Comparison between CPE and Flavourzyme 500 MG®

In order to compare the CPE and Flavourzyme 500 MG®, both extracts were characterized in terms of pH and temperature and optimal activity values and stability using casein (2%) as substrate; additionally degree of hydrolysis (DH) was determined on fish muscle (*E. morio*) as means of fish muscle hydrolytic specificity.

3.2.1. Effect of pH and temperature on enzyme activity

The extracts were active within a wide range of pH 6–10 and temperature (30–60 °C). Non-significant difference ($\alpha = 0.05$) was found in maximum enzyme activity at pH 8 and 9 for CPE (Fig. 2a) while for Flavourzyme 500 MG® the optimal activity was at pH 8 (Fig. 2b). Both extracts exhibited optimal enzyme activity at 50 °C (Fig. 2a and b). Results for optimal pH and temperature of CPE are in agreement with Samarntarn, Cheevadhanarak, and Tanticharoen, (1999) who produced alkaline proteolytic extract by *A. oryzae* U1521 from defatted soybean as a nitrogen source with optimum pH between 8 and 9 and optimum temperature at 45 °C. Boer and Peralta (2000) reported the highest proteolytic activity for *Aspergillus tamarii* from pH 6 to 10, with optimum temperature at 45 °C. Maximum activity was found at pH 8 and 45 °C in proteolytic extracts from *Aspergillus flavus* (Hossain, Das, Marzan, Rahman, & Anwar, 2006). *Monascus purpureus* CCRC31499 produced a protease from shrimp and crab shell powder (SCSP) of marine wastes with optimal pH from 7 to 9 and optimal temperature at 4 °C (Liang, Lin, Yen, Wang, & Wang, 2006). The protease produced by *Thermoascus aurantiacus* showed optimally activity at pH 5.5 and 60 °C (Merheb, Cabral, Gomes, & Da-Silva, 2007). Properties of CPE are no just similar to Flavourzyme 500 MG® but also to some other microbial and commercially available proteases reported before (De Barros, Marques, Melchionna, & Záchia, 2000; Gupta et al., 2002b; Smyth & FitzGerald, 1998).

3.2.2. Effect of pH and temperature on enzyme stability

The effect of pH on enzyme stability was studied at different pH values at 50 °C for 2 h. The CPE maintained over 80% of its original activity between pH 6 and 10 (Fig. 3a), while Flavourzyme 500 MG® retained above 80% of its original activity from pH 6 to 9 (Fig. 3b). Interestingly, CPE retained around 90% of initial activity

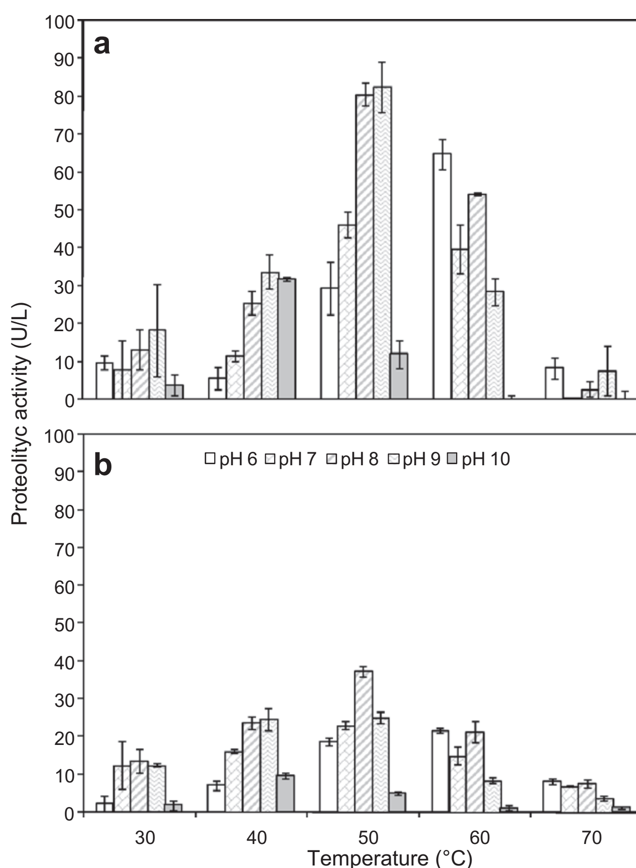

Fig. 2. Effect of pH and temperature on activity of (a) CPE and (b) Flavourzyme 500 MG®.

at pH 10, whereas Flavourzyme 500 MG® retained only 20% of activity. Residual activity values higher than 100% were observed at pH 6 and 7 for CPE and Flavorzyme MG®, respectively; similar results have been recently reported during a characterization of a proteolytic extract from ripe fruit (Vallès, Furtado, & Cantera, 2007). A protease produced by *M. purpureus* CCRC31499 was stable at pH 5–9 (Liang, Lin, Yen, Wang, & Wang, 2006). *T. aurantiacus* produces a proteolytic extract stable in the pH range 3.0–9.5 for 1 h (Merheb, Cabral, Gomes, & Da-Silva, 2007).

The effect of temperature on enzyme stability was determined at different temperatures at pH 8 for 2 h and residual activity was assayed under standard conditions. As shown in Fig. 4a, CPE maintained 100% of activity after 2 h at 30 and 40 °C; however residual activities were 22, 13 and 2% after incubation at 50, 60 and 70 °C, respectively. In contrast, residual activity of Flavourzyme 500 MG® was 60 and 50% at 30 and 40 °C, correspondingly; while at 50, 60 and 70 °C proteolytic activity was almost inactivated (Fig. 4b). The thermal stability of CPE was higher than Flavourzyme 500 MG® at most temperatures assayed. Thermal stability up to 50 °C was reported for a metalloprotease from *A. fumigatus* (Markaryan, Morozova, Yu, & Kolattukudy, 1994). The proteolityc extract from *A. fumigatus* was totally inactivated after 30 min incubation at 60 °C (Wang, Chen, Wang, Yen, & Chern, 2005b). The secondary structure of the protease produced by *Nesterenkonia* sp was unaffected at 50 °C (Bakhtiar et al., 2002). Temperature stability at 50 °C for 15 min from alkaline protease from *Bacillus pumilus* DSM 5777 was reported (Vetter Roman, Bernhard Moeller, & Ingo Muecke, 1994). Complete inactivation occurred above 60 °C for neutral protease from *A. oryzae* (Bombara, Pilosof, & Añón, 1994). Proteases from *M. purpureus* CCRC31499 were stable at 40 °C (Hossain et al., 2006). *T. aurantiacus* produces

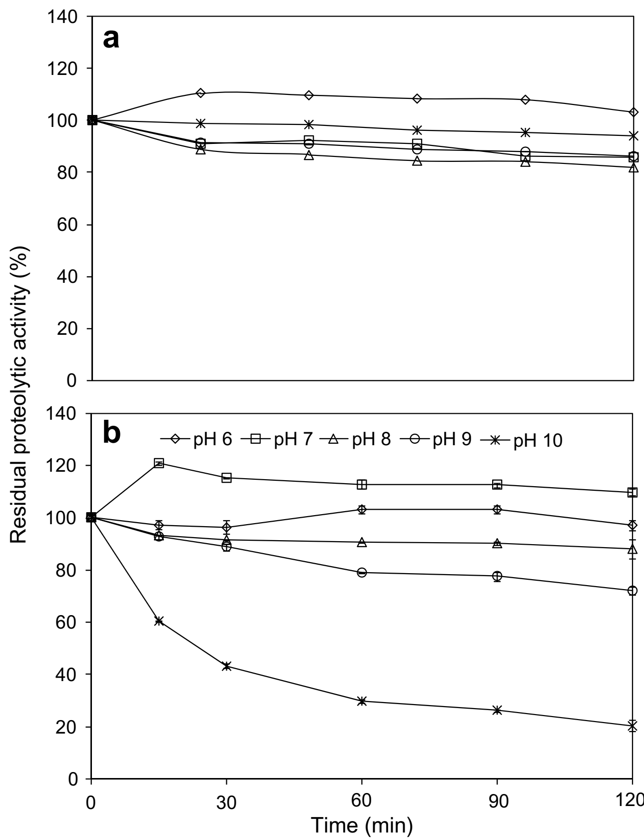

Fig. 3. Effect of pH on stability of (a) CPE and (b) Flavourzyme 500 MG®.

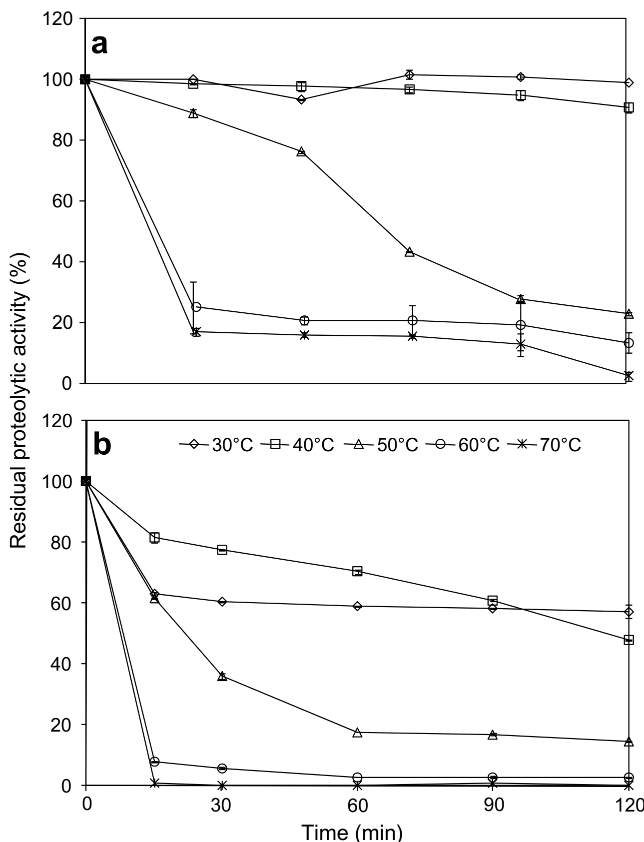

Fig. 4. Effect of temperature on stability of (a) CPE and (b) Flavourzyme 500 MG®.

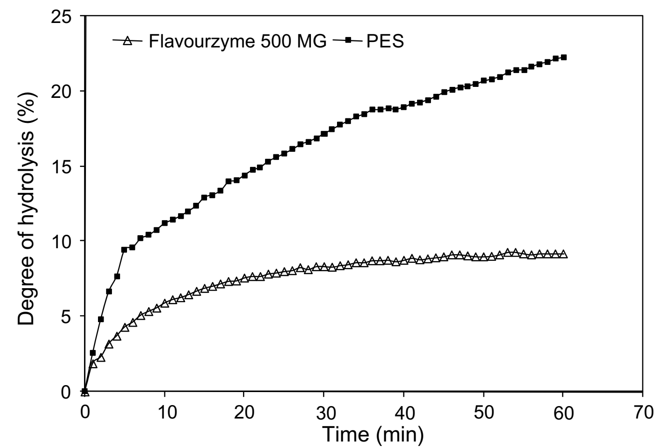

Fig. 5. Degree of hydrolysis of giant sea bass by CPE (■) and Flavourzyme 500 MG® (△) at pH 8 and 50 °C.

a proteolytic extract stable up to 60 °C for 1 h (Merheb et al., 2007). Thermal stability of the CPE showed to be higher than the stability of the proteolityc extracts mentioned above.

The values of half-live time ($t_{1/2}$) at 50 °C for CPE and Flavourzyme 500 MG® were 52 and 25 min, respectively. Similar results were observed for proteases from *A. tamarii*, which presented $t_{1/2}$ values of 75 and 20 min at 50 and 55 °C, respectively (Boer & Peralta, 2000). Based on temperature and pH optimal activity and stability, results showed the suitability of CPE for biotechnological applications where temperatures and pH values ranging from 30 to 55 °C and from 6 to 10, respectively are required.

3.2.3. Degree of hydrolysis (DH)

Kinetics hydrolysis (60 min) for CPE and Flavourzyme 500 MG® on giant sea bass is shown in Fig. 5. Proteolytic activity in CPE presented 2.5 times higher DH (22.2%) than Flavourzyme 500 MG® (9.1%). Similar DH patterns have been reported for fish protein hydrolysis (FPH) from Atlantic salmon (Kristinsson & Rasco, 2000a; Kristinsson & Rasco, 2000b), gold carp (Sumaya-Martínez, Castillo-Morales, Favela-Torres, Huerta-Ochoa, & Prado-Barragán, 2005), yellow tuna wastes (Guèrard, Dufossè, de La Broise, & Binet, 2001) and herring (Liceaga-Gesualdo & Li-Chan, 1999). Similar DH results were reported for Atlantic salmon when the proteases from its own pyloric extract were compared with four commercial proteases (Kristinsson & Rasco, 2000a). In addition, DH obtained with CPE was higher than those reported previously for FPH (Guèrard et al., 2001; Kristinsson & Rasco, 2000a; Kristinsson & Rasco, 2000b). Higher DH levels for CPE could be enhanced since fish flour was used as substrate for CPE production, then higher specificity to fish muscle was obtained.

The results discussed above show that SSF system is a feasible choice to produce CPE by *A. oryzae* 2095 from fish processing by-products, with the advantage that higher pH and temperature stability and DH was achieved when compared to Flavourzyme 500 MG®.

4. Conclusions

The CPE from *A. oryzae* 2095 showed pH and temperature optimum and stability and DH values similar and at times better to microbial and commercial proteolytic extracts previously reported. Additionally the use of fish processing by-products as low-priced substrate for protease production may reduce considerably the cost of the process where protein hydrolysis is involved.

Acknowledgement

García-Gómez acknowledges the scholarship from the National Council for Science and Technology (CONACYT), Mexico.

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