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Extraction of harp seal gastric proteases and their immobilization on chitin

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Gastric proteases from seal stomach were isolated following homogenization, extraction, centrifugation and ammonium sulfate precipitation/fractionation. The crude seal gastric proteases (SGP) were stable in acid conditions with optimum stability at pH 3·0, but were unstable in the alkaline range. The crude SGP possessed excellent temperature adaptability with relative activities of 70 and 90% at 5 and 25°C, respectively. The activity of crude SGPs was retained up to about 40 min incubation at 70°C. The isolated SGP were immobilized on glutaraldehyde-treated chitin. The immobilized SGP exhibited optimum performance at pH 2·0, were most stable at pH 4·0 and had a 90 h half-life in a column with continuous operation for haemoglobin hydrolysis at room temperature. The native SGP clotted milk rapidly at pH 5·8 to 6·6; however, immobilized SGP had a lower milk-clotting activity.

INTRODUCTION

The use of enzymes in the food industry is widespread. Theoreticaly, enzymes can be used in almost all food processing operations in which a biochemical or chemical reaction takes place. However, of the numerous enzymes described, only a mere handful are actually used commercially in the food industry. This is due to reasons such as unsuitable reaction conditions, instability of the enzyme during processing, or the prohibitive costs involved in obtaining large amounts of sufficiently pure enzymes.

Among the enzymes employed in food processing, proteases are most extensively used for improving the quality, stability and functionality of products. Most proteases used in the food industry are derived from microbial and animal sources. In recent years, interest has been expressed in isolating enzymes from marine species. The marine enzymes possess unique physical, chemical and catalytic properties as reviewed by Mohr (1980), Haard *et al.* (1982), Simpson & Haard (1987), Stefansson (1988) and Haard (1992). Novel applications have also been explored (Stefansson & Steingrimsdottir, 1990; Han, 1993).

Proteases from various sources differ greatly in their catalytic and physical properties. Proteases from marine species, especially those from the Atlantic ocean, have unique properties with respect to ease of heat denaturation, high molecular activity at low temperatures and ability to catalyse the hydrolysis of native proteins (Simpson & Haard, 1987). In addition, a relatively narrow specificity of some digestive enzymes from marine environments may avoid the production of bitter peptides during protein hydrolysis, thus serving as good candidates for the production of functional proteins (Han, 1993).

With the development of genetic engineering techniques, it is now possible to clone the gene for an enzyme with unique properties (e.g. from a marine species) and to express it by fermentation using an appropriate bacteria. Recent developments in protein engineering have encouraged biochemists to find special enzymes from various sources. Proteases from harp seal have been investigated previously (Shamsuzzman & Haard, 1983, 1984). The present study was designed to establish a simple procedure for the isolation of seal gastric proteases (SGP), to immobilize the crude enzymes on glutaraldehyde-treated chitin in order to facilitate the control of hydrolysis, to investigate properties of the enzymes so obtained and to explore their potential in food processing.

MATERIALS AND METHODS

Materials

Seal viscera were collected from animals (< 1 year old) hunted off the coasts of Newfoundland, and were frozen immediately until used. The whole viscera were thawed and the stomach was separated, cleaned, ground and subsequently vacuum-packed. The pretreated seal stomach samples were stored at –60°C until

used. Casein was obtained from BDH Chemicals (Toronto, ON, Canada). Glutaraldehyde, haemoglobin (washed and dialysed), calf chymosin (E.C.3.4.23.4), as well as all other chemicals were obtained from Sigma (St. Louis, MO, USA).

Isolation of gastric proteases

The ground frozen stomach was thawed (10 g) and homogenized with ice-cold extraction buffer (60 ml, 0·1 M acetate containing 0·1 M NaCl, pH 4·0) in a Waring blender. The slurry was stirred for 1 h at 22°C and centrifuged at 13 200 *g* for 30 min at 4°C. The supernatant was fractionated, using 20–60% ammonium sulphate, followed by centrifugation at 39 200 *g* for 20 min at 4°C. The enzyme preparations so obtained were dialysed overnight against 0·1 M acetate buffer (pH 4·0) to isolate crude SGP.

Protein determination

Protein was determined according to the method of Lowry *et al.* (1951) or by measuring the absorbance of protein solution at 280 nm during column chromatography, using 0·1 M acetate buffer (pH 3·0) as a blank. Bovine serum albumin was used as a standard.

Enzyme immobilization

One gram of chitin (mesh 7–16), prepared from shrimp shells (Shahidi & Synowiecki, 1991), was treated with 60 ml of a 5·0 M KOH solution at 22°C for 1 h. The KOH-treated chitin was washed sequentially with 1000 ml water and 500 ml of a 0·1 M NaCl solution. The resulting material was suspended in 100 ml of a solution which was 0·1 M in acetate buffer (pH 4·0) and 5 mM in $CaCl_2$, followed by the addition of 100 ml of a 4·0% glutaraldehyde solution. The mixture was kept at 22°C for 20 h. The glutaraldehyde-treated chitin was packed into a 15 cm × 1 cm column. Five millilitres of the crude enzyme solution (39·6 mg enzyme per ml) and 5 ml of a pH 4·0, 0·1 M acetate buffer/5 mM $CaCl_2$ was introduced at the top of the column. The column was left standing for 24 h at 4°C and then washed with 500 ml of the same buffer.

The degree of immobilization (DI) of SGP was calculated using eqn (1):

$$\mathrm{DI}(\%) = \frac{E_a - E_b - E_c}{E_a} \times 100\% \qquad (1)$$

where E_a is the total proteolytic activity of the enzymes in solution before immobilization, E_b is the remaining proteolytic activity in solution after immobilization, and E_c is the proteolytic activity in the washing solution.

Enzyme activity assays

Proteolytic activity using a haemoglobin substrate
Proteolytic activity towards the hydrolysis of haemoglobin was determined using a modified version of the method described by Ryle (1970). The reaction mixture was composed of 0·2 ml enzyme solution, 1·0 ml of 0·2 M acetate buffer (pH 3·0), and 0·6 ml of a 1·5% (w/v) haemoglobin solution. The haemoglobin solution was acidified to pH 1·8 using a 1·0 M HCl solution prior to experimentation. The reaction was stopped by addition of 2·5 ml of 5·0% (w/v) trichloroacetic acid (TCA) solution after exactly 10 min reaction at 25°C. Appropriate blanks were employed by adding TCA to the enzyme prior to the addition of substrate. After 30 min standing at room temperature, the solution was filtered through a Whatman No. 3 filter paper, and the absorbance of the TCA-soluble material was read at 280 nm. One unit of haemoglobin activity (HU) was defined as the amount of protease that increased the absorbance of TCA-soluble haemoglobin hydrolysate by 0·001 unit min at 280 nm under the above given experimental conditions.

Proteolytic activity using a casein substrate
Proteolytic activity using a casein substrate was determined by employing conditions described for the haemoglobin substrate. One unit of casein activity (CU) was defined as the amount of proteases that increased the absorbance of TCA-soluble casein hydrolysate by 0·001 unit/min at 280 nm under the above given experimental conditions.

Immobilized proteolytic units (PU)
Determination of the proteolytic activity of immobilized enzymes was similar to that used for soluble proteases with slight modification. To 500 mg of immobilized protease, 8·0 ml of 0·2 M acetate buffer (pH 3·0) and 4·0 ml of a 1·5% haemoglobin solution was added. The mixture was allowed to stand for 30 min at 25°C with stirring. One millilitre of the supernatant was pipetted into a test tube containing 2·5 ml of 5% TCA solution. The absorbance of the TCA-soluble products was read at 280 nm using the same procedure described above. One immobilized proteolytic unit (IPU) was defined as the amount of enzyme necessary to increase the absorbance of the TCA-soluble products by 0·001 unit/min at 280 nm.

Operational stability of immobilized enzymes
The operational stability of immobilized enzymes was determined by continuous hydrolysis of haemoglobin (0·2%, w/v) in 0·1 M acetate buffer (pH 3·0) in a column as described earlier. The protein solution was passed through the column at a rate of 6 ml/h, and the hydrolysates were collected at the outlet of the column and analysed for their content of TCA-soluble products. Losses in proteolytic activity were determined by monitoring changes in the TCA-soluble products from the hydrolysis of proteins.

Milk-clotting activity (MCA)
The MCA of SGP was assayed using the procedure of Manji *et al.* (1988) with minor modifications. One gram of immobilized enzyme (0·2 ml of soluble proteases,

100–500 HU/ml) was added to a 30 ml of reconstituted milk consisting of 12% Carnation instant skim milk powder and 0·01 M $CaCl_2$ at pH 6·2. The mixture was then gently and constantly swirled until the first appearance of a white precipitate at the bottom of the beaker. The time for first appearance of precipitate was taken as the milk-clotting time (MCT). One milk-clotting unit (MCU) was defined as the amount of enzyme that clotted 10 ml of reconstituted milk in 100 s at 25°C (Manji *et al.*, 1988).

pH optima and stability

The effect of pH (1·4–8·5) on the hydrolysis rates of casein and haemoglobin was determined using various buffers. The procedures employed for measuring the rate of hydrolysis were the same as those described above. To determine pH stability, proteases (native or immobilized) were incubated in various buffers under different pH conditions for 24 h at 25°C prior to determination of the residual activities according to the methods described above. Buffers were prepared according to Teorrell & Stenhagen (1938).

Temperature optima and thermal stability

The effect of temperature variation on the hydrolysis rate of casein and haemoglobin was determined by performing complete hydrolysis at 5–80°C. Proteases were incubated at various temperatures for different time intervals prior to determination of the residual activities in order to examine their heat stability.

RESULTS AND DISCUSSION

Table 1 summarizes the results of the extraction of proteolytic enzymes from seal stomach. Seal stomach (1 kg) yielded *c.* 12 g of crude gastric proteases with 25% recovery of proteolytic activity. The total PU include both zymogens and activated proteases due to the extraction conditions employed.

The crude soluble proteases showed a pH optimum of about 3·0 for haemoglobin as substrate over a 20 min incubation period at 25°C (Fig. 1(A)). It has been reported that many fish species secrete at least two pepsins with different pH optima (Noda & Murakami, 1981; Gildberg & Raa, 1983) which cover a broad pH range at *c.* 3·0 for haemoglobin (Donta & Van Vunakis,1970; Sanchez-Chiang & Ponce, 1981).

The stability of proteases in buffers at various pH

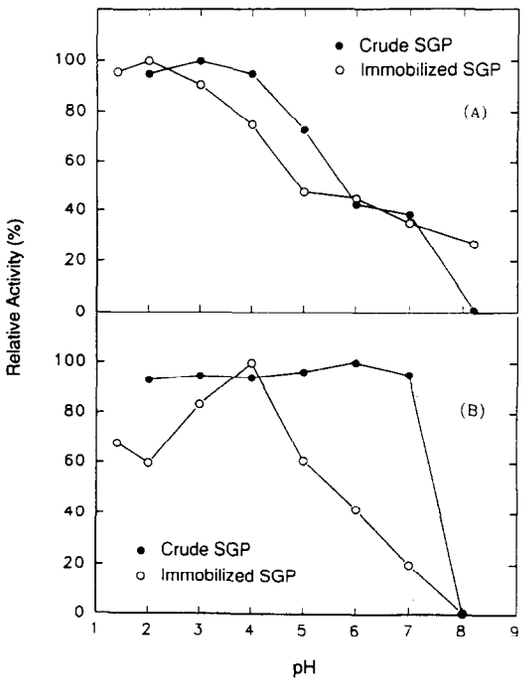

Fig. 1. Influence of pH on relative activity of crude soluble and immobilized SGP: (A), for 0·5% haemoglobin at 25°C after 20 min; (B), the same as (A), but after 24 h incubation at 25°C. Residual activity was measured at pH 3·0 in 0·2 M acetate buffer after a 20 min digestion of 0·5% haemoglobin at 25°C.Results are mean values of triplicate determinations.

conditions after 24 h incubation at 25°C is shown in Fig. 1(B). The isolated proteases were stable in acid, but unstable under alkaline conditions. Total loss of activity was noted at pH >8·0 (Fig. 1(B)). This is similar to the behaviour of gastric proteases from fish species. However, at pH 7·0, the crude SGP exhibited >90% of their maximum activity.

The effect of temperature on the activity of crude SGP was considerably different from other proteases. The crude SGP exhibited 70% of their activity at temperatures as low as 5°C and around 90% at 25°C (Fig. 2). Fish pepsins exhibit low thermal stability and show great molecular flexibility, hence optimum activity at low temperatures (Gildberg, 1988). Generally, the thermal stability is closely related to the temperature of optimum activity. The temperature for optimum activity of an enzyme is generally slightly below its denaturation temperature (Foltmann, 1981). However, the crude SGP survived for more than 40 min at 70° (Fig. 3) and had a temperature optimum close to 45°C when

Table 1. Isolation of harp seal gastric proteases[a]

Purification step	Activity[b] (HU/ml)	Protein content (mg/ml)	Specific activity (HU/mg)	Total volume (ml)	Recovery (%)
Supernatant from 1st centrifugation	204	12·4	16·5	1150	100
Precipitated with 20% $(NH_4)_2SO_4$	151	8·5	17·8	1140	73·4
Precipitated with 60% $(NH_4)_2SO_4$	985	39·6	23·9	60	25·2

[a]From 200 g seal stomach; HU: haemoglobin activity unit.
[b]The total proteolytic activity was measured using haemoglobin as a substrate. Results are mean values of triplicate determinations.

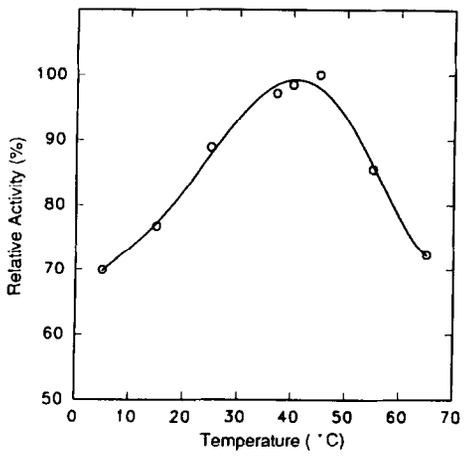

Fig. 2. Influence of temperature on the proteolytic activity of crude SGP after 20 min incubation in 0·2 M acetate buffer, pH 3·0. Results are mean values of triplicate determinations.

haemoglobin was used as the substrate (20 min incubation at pH 3·0; Fig. 2). In addition, the present study indicated the absence of any apparent relationship between temperature optima and thermal stability of SGP. The temperature optimum for enzyme extracts from Arctic capelin, North Sea herring and Indian oil sardine was 45°C (Gildberg, 1982). The temperature adaptability of SGP indicated that the enzyme system possessed characteristics of both fish and mammal pepsins. Therefore, SGP showed an optimum activity at 45°C similar to that for crude fish pepsin and were as highly thermostable as other mammalian pepsins. The wide activity range of SGP under different temperature conditions makes them attractive candidates for commercial applications and for investigations on enzyme reaction mechanisms.

Immobilization of crude SGP and properties

Activity retention of crude SGP immobilized on chitin after washing was about 20–29% of that of the enzyme prior to immobilization. The average DI was around 24%, corresponding to a binding of *c.* 4 mg of enzyme per 100 mg of chitin. However, further research is required to optimize many factors which may affect the immobilization conditions.

The immobilized SGP showed an optimum pH of approximately 2·0 when using haemoglobin as the substrate (Fig. 1(A)), which is typical for pepsin (Ward *et al.*, 1978). Therefore, the immobilized SGP are considered as being pepsin-like in nature. Furthermore, comparison of the present results with those of Shamsuzzaman & Haard (1984) indicates that immobilized SGP belong to the protease C group as classified by these investigators. However, the pH profile may not be a reliable criterion for distinguishing gastric proteases, especially for immobilized enzymes where shifting of pH profiles compared to native enzymes may occur.

The immobilized SGP system was stable over a relatively narrow pH range and was most stable at pH 4·0. In the acid range, it lost considerable activity even at the optimum pH. This suggests that the crude preparation may contain some stabilizing factors. The immobilization of enzymes may also limit their conformational mobility, thus influencing their activity.

Operational and storage stability of immobilized SGP

The operational stability of the immobilized SGP was evaluated using a packed column for hydrolysis of protein solutions. Variations of the absorbance of TCA-soluble haemoglobin hydrolysates at 280 nm over a 100 h reaction period is shown in Fig. 4. After the initial rise in activity, the absorbance values of the hydrolysate decreased slowly and progressive inactivation took place after prolonged operation of the column. No soluble protease activity was measured in the solution of haemoglobin hydrolysate.

Samples of immobilized SGP were stored as a wet cake in a 0·1 M acetate buffer at pH 4·0. The storage stability of the protease–chitin complex was determined by periodically assaying for the proteolytic activity of

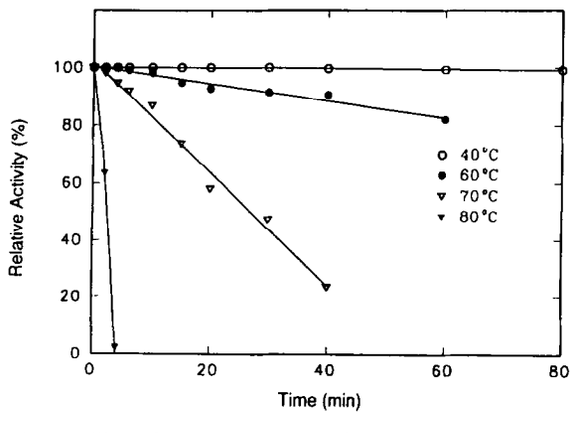

Fig. 3. Stability of crude SGP at various temperatures. Residual activity was measured after 20 min incubation with 0·5% haemoglobin in 0·2 M acetate buffer pH 3·0 at 25°C. Results are mean values of triplicate determinations.

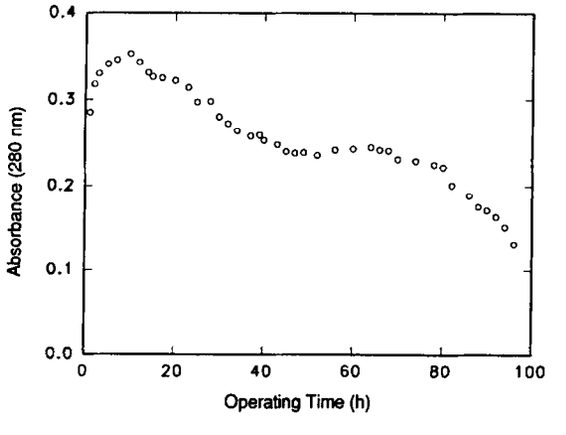

Fig. 4. Residual enzymic activity (absorbance) of immobilized SGP as a function of operation time under continuous hydrolysis conditions. A 0·2% haemoglobin solution in 0·1 M acetate buffer, pH 3·0, was continuously hydrolysed at ambient temperatures.

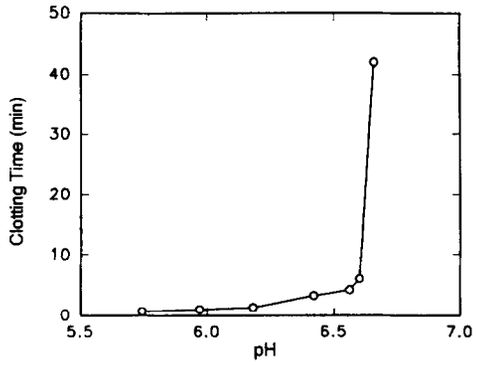

Fig. 5. Influence of pH on milk-clotting time by crude SGP. The milk was adjusted to different pH values using a 1·0 M HCl or 1·0 M NaOH solution. Results are mean values of triplicate determinations at each pH.

the immobilized SGP. Results indicated that the immobilized SGP system was quite stable when stored as a moist cake at 5°C. Only about 7% of its activity was lost over 120 days storage at 5°C; however, when stored in buffer solutions, *c.* 45% of its activity was lost under similar conditions and over the same period.

Catalytic activity of immobilized SGP

The crude native SGP clotted milk rapidly at pH 5·8 to 6·6 (Fig. 5). Since pepsin is unable to clot milk at high pH values (Gordin & Rosenthal, 1978) the crude SGP preparation has some similarity to chymosin. However, the immobilized SGP had a somewhat lower MCA compared with its non immobilized counterpart (Table 2). It has been shown that all aspartic proteases have similar folding of their peptide chains and that the pepsin molecule is bilobal with the two domains separated by a deep cleft perpendicular to the largest diameter of the molecule (Foltmann, 1981). The highly flexible conformation of the molecule is important for its effective catalytic activity. However, immobilization greatly limited the conformational flexibility and hence lowered the activity of the enzyme.

Crude and immobilized SGP showed different proteolytic activities with various substrates (Figs 6 and 7). The hydrolysis rate of casein at pH 3·0 was much faster than that at pH 6·0. Shamasuzzaman & Haard (1983) indicated that the hydrolysis of casein, catalysed by

Table 2. Proteolytic properties of SGP towards different substrates[a]

Enzyme	HU (mg)	CU (mg)	MCU (mg)	MCU/ HU	MCU/ CU
Native crude SGP	24·9	18·1	0·49	0·020	0·027
Immobilized SGP	9·5	3·6	0·10	0·011	0·028
Calf chymosin	34·9	61·4	5·17	0·148	0·084

[a]Data for HU (haemoglobin activity unit) and CU (casein activity unit) are based on the initial velocities of the reaction after 10 min incubation at 25°C. The pH for HU and CU was 3·0, and for the milk-clotting unit (MCU) was 6·18. All other conditions were the same as those in the text. Results are mean values of triplicate determinations.

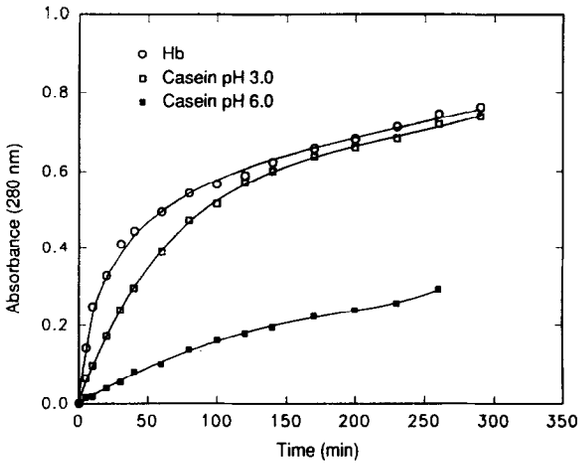

Fig. 6. Proteolytic activity (absorbance) of crude native SGP towards casein and haemoglobin. Samples were incubated for 20 min at 25°C at a 0·5% concentration of substrate. Results are mean values of triplicate determinations.

porcine pepsin, declined after 60 min compared with the SGP-catalysed reaction, which took 5 h without showing any decline (Fig. 7).

The crude native SGP showed a higher proteolytic activity towards casein compared with its immobilized counterpart. This indicated that some proteases with higher activity towards casein may not have been immobilized. However, under immobilized conditions, intimate contact between enzyme and substrate becomes more difficult due to steric hindrance. Less conformational flexibility of the immobilized proteases may contribute to their lower proteolytic activity towards substrates.

Proteolytic properties of crude and immobilized SGP are summarized in Table 2. The results are consistent with those reported by Shamsuzzaman & Haard (1983), except for the ratio of MCU to PU, perhaps due to the existing differences in the pH activity of enzyme for casein hydrolysis. The ratio of MCU/PU has been used to determine the suitability of isolated proteases in cheese making (Puhan & Irvin, 1973; deKoning *et al.*,

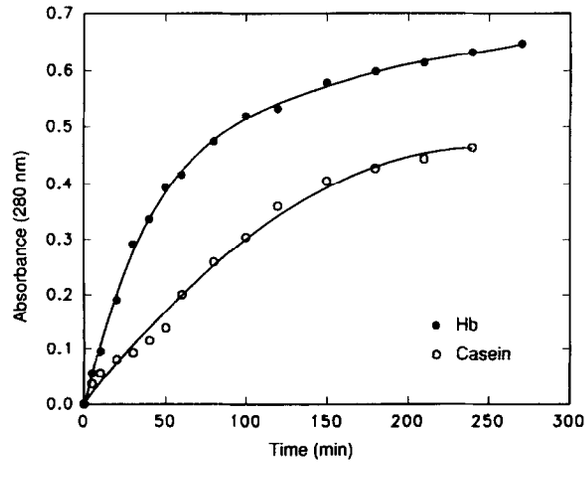

Fig. 7. Proteolytic activity (absorbance) of immobilized SGP, using casein and haemoglobin. Conditions are the same as those given in the caption to Fig. 3.

1978). A high value of MCU/PU is indicative of proteases which are successfully used in cheese making (Visser, 1981). The crude SGP preparation showed a higher MCUIHU ratio than that of its immobilized counterpart (0·020 vs 0·011). In addition to changes in conformational flexibility, it is possible to postulate that the main fraction of the immobilized SGP is acid protease C.

CONCLUSIONS

Seal stomach provided an excellent source of proteases with potential application in protein hydrolysis and as a possible substitute for milk clotting. The characteristics of the crude native SGP were somewhat similar to those of chymosin. However, mainly pepsin-like enzymes were immobilized on chitin. The crude SGP immobilized on chitin were active over a 3 day period under continuous operation at room temperature.

REFERENCES

deKoning, P. J., van Rooijen, P. J. & Visser, S. (1978). Application of a synthetic hexapeptide as a standard substrate for the determination of the activity of chymosin. *Neth. Milk Dairy J.*, **32**, 232–44.

Donta, S. T. & Van Vunakis, H. (1970). Chicken pepsinogens. *Methods Enzymol.*, **19**, 358–63.

Foltmann, B. (1981). Gastric proteases—structure, function, evolution and mechanism of action. *Assays Biochem.*, **17**, 52–84.

Gildberg, A. (1982). Autolysis of fish tissue — general aspects. Thesis. Institute of Fisheries, University of Tromso, Norway.

Gildberg, A. (1988). Aspartic proteinases in fishes and aquatic invertebrates. *Comp. Bioche. Physiol.*, **91B**, 425–35.

Gildberg, A. & Raa, J. (1983). Purification and characterization of pepsins from the Arctic fish capelin (*Mallotus villous*). *Comp. Biochem. Physiol.*, **75A**, 337–42.

Gordin, S. & Rosenthal, I. (1978). Efficacy of chicken pepsin as a milk clotting enzyme. *J. Food Protect*, **41**, 684–8.

Haard, N. F. (1992). A review of proteolytic enzymes from marine organisms and their utilization in the food industry. *J. Aquatic Food Prod. Technol.*, **1**, 17–35.

Haard, N. F. Feltham, L. A. W., Helbig, N. & Squires, E. J. (1982). Modification of Proteins with proteolytic enzymes from the marine environment. In *Modification of Proteins*, ed. R. E. Feeney & J. R. Whitaker. *Advances in Chemistry Series* 198. American Chemical Society, Washington, DC, pp. 223–44.

Han, X.-Q. (1993). Recovery of digestive enzymes from Atlantic cod (*Gadus morhua*) and their utilization in food processing. MSc Thesis. Memorial University of Newfoundland.

Lowry, O. H. Rosebrough, N. G., Farr, A. L. & Randall, R. J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

Manji, B., Yada, R. Y., Findlay, C. J. & Parkin, K. L. (1988). Optimization of the immobilization of milk-clotting proteases to granular bone. *Food Biotechnol.*, **2**(1), 43–65.

Mohr, V. (1980). Enzyme technology in the meat and fish industries. *Proc. Biochem.*, **21**(8/9), 18–21, 32.

Noda, M. & Murakami, K. (1981). Studies on proteinases from the digestive organs of sardine. Purification and characterization of two acid proteinaese from the stomach. *Biochem. Biophys. Acta*, **685**, 27–34.

Puhan, Z. & Irvine, D. M. (1973). Proteolysis by proteases of *Bacillus subtilis* used to make Canadian cheddar cheese. *J. Dairy Sci.*, **56**, 317–24.

Ryle, A. P. (1970). The porcine pepsins and pepsinogens. In 'Methods in Enzymology, *ed. G. E. Perlmann, S. P. Colowick & N. O. Kaplan. Academic Press, London, Vol. XIX, pp. 316–36.*

Sanchez-Chiang, L. & Ponce, O. (1981). Gastricsinogens and gastricsins from *Merluccius gayi* — purification and properties. *Comp. Biochem. Physiol.*, **68B**, 251–7.

Shahidi, F. & Synowiecki, J. (1991). Isolation and characterization of nutrients and value-added products from snow crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *J. Agric. Food Chem.*, **39**, 1527–32.

Shamsuzzaman, K. & Haard, N. F. (1983). Evaluation of harp seal gastric protease as a rennet substitute for cheddar cheese. *J. Food Sci.*, **4**, 179–82.

Shamsuzzaman, K. & Haard, N. F. (1984). Purification and characterization of a chymosin like protease from the gastric mucosa of harp seal (*Pagophilus groenlandius*). *Can. J. Biochem. Cell Biol.*, **62**, 699–708.

Simpson, B. K. & Haard, N. F. (1987). Cold-adapted enzymes from fish. In *Food Biotechnology*, ed. D. Knorr. Marcel Dekker, New York, pp. 495–528.

Stefansson, G. (1988). Enzymes in the fish industry. *Food Technol.*, **42**(3), 64–65.

Stefansson, G. & Steingrimsdottir, G. (1990). Application of enzymes for fish processing in Iceland — present and future aspects. In *Advances in Fisheries Technology and Biotechnology for Increased Profitability*, ed. M. N. Voigt & J. R. Botta. Technomic, Lancaster (USA) and Basel (Switzerland), pp. 237–50.

Teorell, T. & Stenhagen, E. (1938). Universal buffer over the pH range 2·0 to 12·0. *Biochem. J.*, **299**, 416–19.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. *Neth. Milk Dairy J.*, **35**(1), 65–72.

Ward, P. H., Neumann, V. K. & Chiang, L. (1978). Partial characterization of pepsins and gastricsins and their zymozens from human and total gastric mucosae. *Comp. Biochem Physiol.*, **61B**, 491–8.