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Purification and characterization of an acidic protease from the viscera of bolti fish (*Tilapia nilotica*)

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Abstract

An acidic protease was extracted from acetone powder of the viscera of bolti fish (*Tilapia nilotica*) in acidified distilled water and precipitated from the resulting extract by ammonium sulfate followed by dialysis and its kinetics studied. The crude enzyme was purified using gel filtration; its homogeneity and molecular weight were studied. The enzyme showed highest activity and purification-fold when precipitated at 40–60% ammonium sulfate. Purification fold and activity were increased after purification by dialysis and gel filtration on Sephadex G-100. Homogeneity studies, by polyacrylamide gel electrophoresis, illustrated that the enzyme was homogeneous only after purification by the gel filtration step. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed a molecular weight of 31.0 kDa. The optimal pH and optimal temperature were 2.5 and 35°C, respectively. The enzyme showed pH stability between 2 and 6. It retained more than 50% of its activity after heating between 50 and 60°C for 30 min, and 40.2 and 74.9% after heating between the same temperatures for 120 min. The K_m and V_{max} values of enzyme were 0.77 mM and 2.22 mM/min, respectively, while the catalysis efficiency (V_{max}/K_m) was 2.88. A high inhibition percentage of total enzyme activity was obtained when the enzyme was incubated with 50 mM of both soybean trypsin inhibitor and ethylenediaminetetraacetic acid. The presence of either NaCl or $CaCl_2$ at 10 mM concentration increased the enzyme activity by 20.5% and 31.2%, respectively.

Keywords: Bolti fish; Viscera; Acidic protease; Extraction; Purification; Kinetic reaction

1. Introduction

Proteases constitute the most important group of industrial enzymes used in the world today and it have several applications in the food industry (Garcia-Carreño, 1991).

Proteases may be extracted from any living organism. Very wide ranges of sources are used for commercial enzyme production (Chaplin & Buck, 1990). Proteases are mainly derived from plant, animal and microbial sources, whereas their counterparts, derived from marine and other aquatic sources, have not been extensively used (Haard & Simpson, 1994).

Fish processing generates large amounts of solid and liquid wastes. Normally, more than half of the raw material weight is unused. For example, only about 15% of round shrimp become a canned shrimp product. Many different by-products have been produced from fish processing wastes. However, fishery by-products are typically feeds and fertilizers that have a low dollar value. There is growing interest in obtaining higher value biochemicals and pharmaceuticals from fishery wastes, notably enzymes (Haard, 1998).

There have been relatively few attempts to use fish proteases as industrial processing aids. Fish are poikilothermic and vary considerably in their feeding habits and temperature preferences, and so it is expected that their digestive enzymes will also exhibit diversity. (Godfrey & Reichelt, 1983).

In recent years, additional applications of proteases in the seafood industry have emerged. These include the selective removal of skin, hydrolysis of membranes and other supportive tissue that envelope roe, roe seeks and other tissues, and recovery of pigments and flavour extract (Haard, 1992). Crude forms of both acidic and alkaline proteases extracted from bolti fish viscera convert 54% and 79% of fish protein wastes at a 50 h reaction time (Ibrahim, 1994).

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This research is carried out as a contribution to the utilization of fish by-products for producing enzymes and also to the reduction of waste disposal problems.

2. Materials and methods

2.1. Materials

2.1.1. Bolti fish viscera

The Bolti fish, *Tilapia nilotica*, were purchased from the local market at Shibin El-Kom city and the viscera were removed by hand as soon as possible, then stored in sealed plastic bags at −20°C until used for enzyme extraction.

2.1.2. Reagents

Bovine serum albumin, trichloroacetic acid, glycerol, β-mercaptoethanol, tris (hydroxymethylaminomethane), Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide, N,N,N,N, tetramethylethylenediamine, sodium dodecyl sulphate, ammonium persulphate and ethylenediaminetetraacetic acid were purchased from Sigma Chemical Co., St. Louis, Mo. USA. Protein standard was purchased from Bio-Rad, Mississauga, Ontario, Canada. Sephadex G-100 and DEAE-sephadex A-25 were purchased from Pharmacia Fin Chemical Co. Uppsala, Sweden. Pepsin powder was purchased from Riedel-de Haën.

2.2. Methods

2.2.1. Preparation of crude acidic protease

Digestive tracts (viscera) were partially thawed in the refrigerator at 4°C, defatted by homogenization with 8 volumes of cold acetone for 30 s in a homogenizer (Edmund Bühler, Tühler, Tübingen, Typ. Hov Nr. 52). The homogenate was filtered and the acetone insoluble material was washed several times with cold acetone and finally once with ether, then dried at room temperature at 25+2°C overnight. The powder produced was kept in a brown glass bottle and stored at −20°C for further analysis.

The acetone dried powder was extracted with distilled water (1: 20, w/v) for 1 h using a mechanical shaker. The resulting supernatant was adjusted to pH 2.5 by addition of 0.1 M HCl then held for 30 min and readjusted to pH 5 with 0.1 M NaOH after removal of the acidic precipitate. The supernatant was dialyzed against distilled water at 4°C overnight (Haard, 1986) and referred to as crude acidic protease.

2.2.2. Enzyme purification

2.2.2.1. Ammonium sulfate precipitation. For preliminary assay, 50 ml of enzyme extract were used to determine the appropriate ammonium sulphate concentration for partial purification. The ammonium sulphate concentrations (40–60%) which gave the highest purification-fold and specific activity were used to purify the remaining initial extract through two steps: first, the solution was brought to 40% concentration then centrifuged at 10,000 rpm for 10 min and the precipitate discarded. Secondly, the enzyme solution was brought to 60% saturation and the enzyme precipitate retained and the remainder discarded. The resulting extract was dialyzed and defined as partially purified acidic protease, and dissolved in 0.02 M acetate buffer at pH 5.0. It was kept in brown glasses bottles and stored at −20°C for further purification.

From the preliminary study, acidic protease was collected from the crude acidic supernatant by precipitation with 40–60% saturation of ammonium sulfate. It was mixed for 30 min, then allowed to settle for 24 h at 4°C. The supernatant was discarded and the precipitate was dissolved in 0.02 M acetate buffer of pH 5.0.

2.2.2.2. Dialysis. The enzyme produced from the previous steps was dialyzed against the same buffer (0.02 M acetate of pH 5.0) for 24 h at 4°C and the buffer was changed twice.

2.2.2.3. Gel filtration. The dialyzed enzyme was fractionated on a Sephadex G-100 column of 1×50 cm. The column was equilibrated and eluted with 0.02 M acetate of pH 5.0. Elution was carried out at a flow rate of 20 ml/h and 3 ml fractions were collected manually. Absorbances at 280 nm and enzyme activity were measured in all fractions using a spectrophotometer (Bausch and Lomb spectronic 2000). The major active fractions were collected and stored at −20°C for further analysis.

2.2.2.4. Determination of enzyme homogeneity. Polyacrylamide gel electrophoresis was used to monitor the enzyme homogeneity as described by Davis (1964). Crude and purified enzymes were mixed with 0.01 M sodium phosphate buffer of pH 7.8 and dialyzed overnight at 4°C against the same buffer. The electrophoresis was performed in 0.01 M sodium phosphate buffer of pH 7.8 at a constant current of 3 ma/tube for 3 h. The gels were stained with 0.5% Amido Black in 7.5% acetic acid for 30 min. Destaining was done in 7.5% acetic acid to remove the free dye.

2.2.2.5. Determination of the molecular weight. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis was carried out to determine the molecular weight of purified enzyme using the method of Kemény et al. (1989). Samples were prepared by mixing the purified enzyme extract with distilled water containing 0.062 M Tris, 4% sodium dodecyl sulphate, 10% glycerol, 1.5% β-mercaptoethanol and 0.002% bromophenol blue (pH

6.8). Polyacrylamide gels (monomer 8.33% concentration) in vertical gel tubes containing 0.15% SDS and 0.375 M Tris were prepared. The running buffer contained 3.03 g Tris, 14.42 g glycine and 1 g SDS per litre. Then, 100 μl of samples were applied on to the gel surface and fractionated for 3 h at 300 V under refrigeration. The gel was stained overnight in 25 mg Coomassie Brilliant Blue-R 250 in trichloroacetic acid, distilled water, methanol and acetic acid (5.8: 72: 18: 6, w/v/v/v), then destained in distilled water; methanol and acetic acid (134: 58: 10, v/v/v). Standard protein calibrants, i.e. phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), kuntize soybean trypsin inhibitor (21,000), were used as molecular weight markers. The relative mobilities were calculated and plotted against the molecular weight logarithm for calculating the molecular weight of proteins.

2.3. Assay of protease activity

The acidic protease activity was determined as described by Anson and Mirsky (1932) using 2% haemoglobin at pH 2 and 30°C. The pepsin unit (PU) were calculated from the initial slope of the A 280 nm time course of the TCA soluble product, as the amount of enzyme that produces an A 280 nm increase of 0.0042 per min per ml of reaction mixture.

2.4. Protein content

Protein content was determined by the method of Lowry, Resebrough, Far and Randell (1951) using bovine serum albumin as standard.

2.5. Enzyme kinetic methods

2.5.1. pH optima

The pH optimum of the protease enzyme was determined by preparing the haemoglobin substrate in various buffer solutions (0.2 M HCl–KCl buffer of pH 2.0, 0.2 M citrate phosphate buffer of pH 3–7 and 0.2 M Tris–HCl buffer of pH 7–11) and applying the enzyme extract to the substrate to assay the enzyme activity.

2.5.2. pH stability

The influence of pH on the stability of the protease was determined by pre-incubating the enzyme in the above mentioned buffer solutions for 30 min at room temperature (25±1°C) then determined the remaining activity.

2.5.3. Temperature optima

The influence of temperature on the activity of the acidic protease was determined at various temperature intervals (10–80°C).

2.5.4. Thermostability

The enzyme solution was incubated at various temperatures (50, 60, 70 and 80°C) for 30, 60, 90 and 120 min, then cooled rapidly in an ice bath for 5 min and the residual activity assayed.

2.5.5. Determination of K_m and V_{max}

K_m and V_{max} of the acidic enzyme were determined using haemoglobin as a substrate at different concentrations (2.5, 5, 7.5 and 10 mg/ ml) and calculated as described by the Lineweaver–Burke (1934) method.

2.5.6. Inhibitors and activators

The effects of some inhibitors, such as soybean trypsin inhibitor and ethylenediaminetetraacetic acid, as well as activators, such as $CaCl_2$ and NaCl at different concentrations (10, 20, 30, 40 and 50 mM), on the enzyme activity were determined by pre-incubation with the substrates for 10 min at the optimum temperature of enzyme. The enzyme was added and the activity was determined as described above. Inhibition and activation of enzyme activity were expressed as a percentage of the activity without modifiers.

3. Results and discussion

3.1. Purification of the acidic protease

'The purification steps, protein concentration, specific activity and yield of acidic protease are shown in Table 1. The specific activity and purification fold were 0.25

Table 1
Purification steps of the acidic protease from Bolti

Purification steps	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	1000	1960	32660	0.06	100	1.00
Ammonium sulphate (40–60%)	60	823	3292	0.25	42	4.16
Dialysis	60	802	3084	0.26	41	4.33
Gel filtration	18	238	216	1.10	12	18.3

$\text{Specific } \frac{\text{Total activity (U)}}{\text{Total protein (mg)}}$ $\%\text{Recovery} = \frac{\text{Total activity}}{\text{Total activity of crude extract}} \times 100$ $\text{Purification-fold} = \frac{\text{Specific activity}}{\text{Specific activity of crude extract}}$.

U/mg protein and 4.16, respectively, when 40–60% ammonium sulfate used.

The collected precipitate from the previous step was dissolved in 0.02 M acetate buffer of pH 5.0 and dialyzed against the same buffer for 24 h at 4°C. The dialysis step increased the specific activity from 0.25 to 0.26 and purification fold from 4.16 to 4.33 (Table 1). Raksakulthai and Haard (1999) reported that dialysis of amino peptidase crude extract (from Squid hepatopancreas) increased the purification fold from 2.08 to 4.6.

The dialyzed enzymes were further purified by applying 1 ml of the dialyzate to a Sephadex G-100 column (1×50 cm) and eluted with 0.02 M acetate buffer of pH 5. Fig. 1 illustrates the fractionation pattern of partial purified acidic protease by gel filtration chromatography. Only one peak was obtained at an elution volume of 24–39 ml and V_e/V_o of 1.98. This active peak has a high specific activity (1.10 U/mg protein) and purification fold (18.3), as shown in Table 1.

The homogeneity of the acidic protease during purification steps is illustrated in Fig. 2. The resulting solution, after ammonium sulfate purification and dialysis, was still not homogeneous, while the polyacrylamide gel electrophoresis pattern of pure acidic protease eluted by column chromatography revealed only one band; this result proved that the enzyme is quite homogeneous.

The molecular weight of the acidic protease eluted by column chromatography was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis. There was a single and destained protein band with

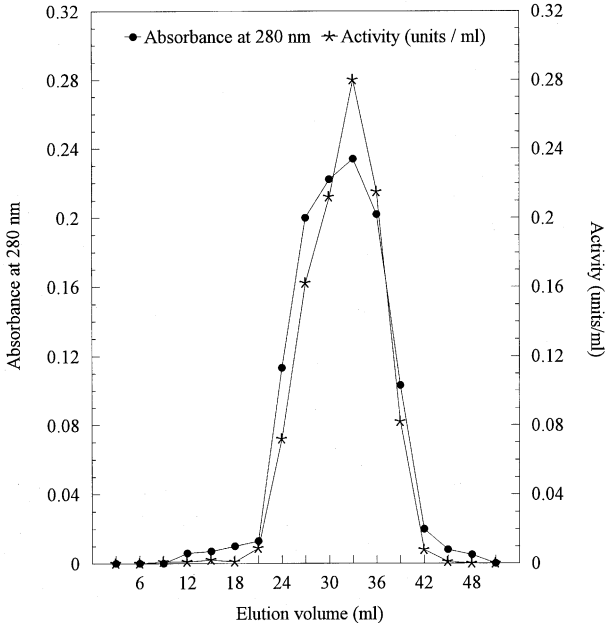

Fig. 1. Gel filtration pattern of the acidic protease of bolti on Sephadex G-100.

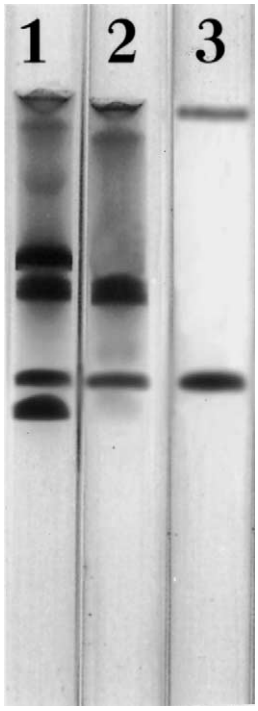

Fig. 2. Homogeneity test of the acidic protease of bolti as determined by polyacrylamide gel electrophoresis. 1. Crude extract. 2. Purification by ammonium sulphate (40–60%). 3. Purification by gel filtration.

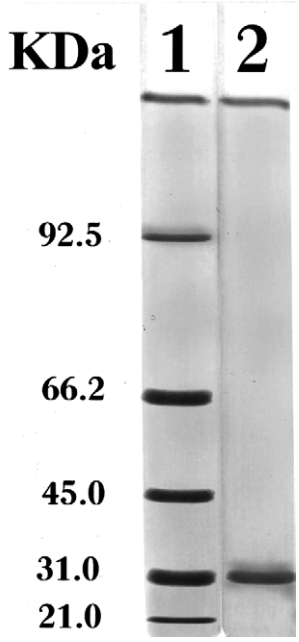

Fig. 3. SDS–PAGE pattern of pure acidic bolti protease. 1. Standard proteins. 2. Pure acidic protease.

R_f corresponding to a molecular weight of 31,000 Dalton (Fig. 3). Lopez-Liorca (1990) reported that the acidic protease had a molecular weight around 32,000 Dalton.

3.2. Enzyme kinetics

3.2.1. pH optima

The partially purified acidic protease had the highest activity at pH 2.5 and it then decreased with increasing of pH (Fig. 4). Over pH 4, more than 50% of the relative activity was lost and no activity was detected at pH 8. The optimum pH for the hydrolysis of haemoglobin by partially purified Polar cod pepsin was found to be 3.2 at 30°C and 2.5 at 5°C (Haard et al., 1982); also crude pepsin had a broad optimum of pH 2–3 at 28°C.

3.2.2. pH stability

Fig. 5 illustrates pH stability of the acidic protease. The acidic protease retained more than 90% of its original activity in the pH range 2–5 and then decreased with increasing pH and reached its lowest relative activity at pH 11 (7%). These data clearly indicate that the acidic protease was most stable in the pH range 2–6 and least stable within the pH range 7–11. The instability of acidic proteases towards the alkaline pH region contrasts with the behaviour of gastric protease of many of the lower vertebrate species (Yamamoto, 1975). Generally, these data are in agreement with that reported by Squires et al. (1986a) for Green land Cod gastric protease and Ibrahim (1994) for bolti acidic proteases.

3.2.3. Temperature optima

Fig. 6 illustrates the influence of different temperatures on the acidic protease activity. The relative activity increased with increasing the temperature from 10 to 35°C and then decreased; however no activity was detected at 70°C. Generally, these data are in agreement with those reported by Simpson, Simpson, & Haard (1990) and Dimes, Garcia-Carreno, & Haard (1994).

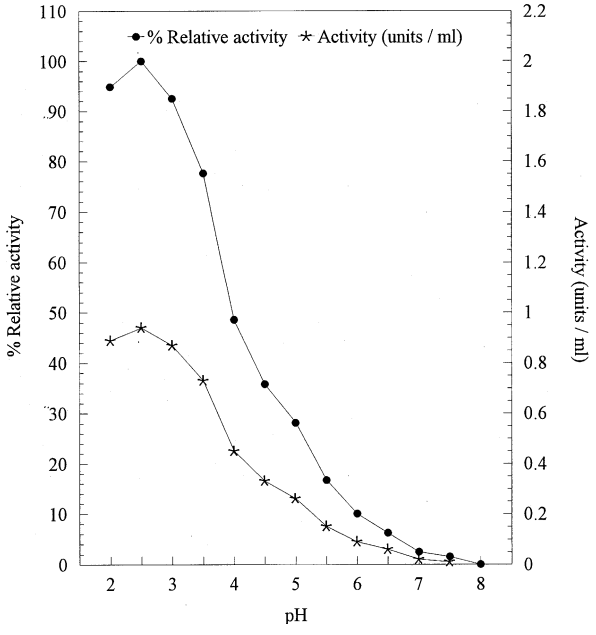

Fig. 4. Effect of pH on the activity of the acidic protease of bolti.

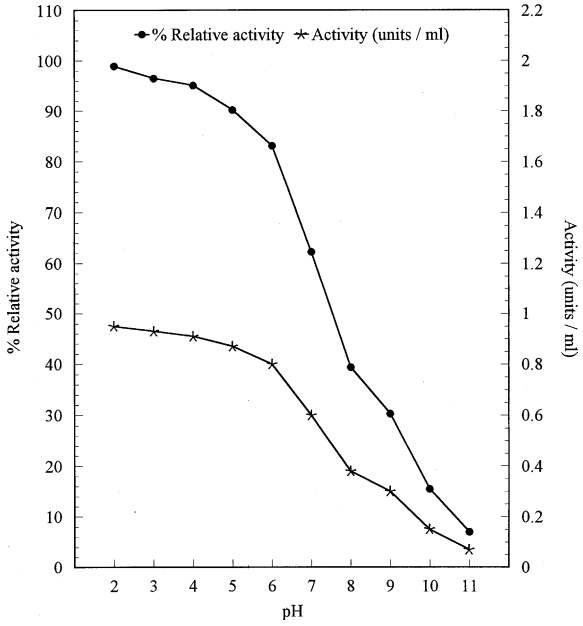

Fig. 5. pH stability of the acidic protease of bolti.

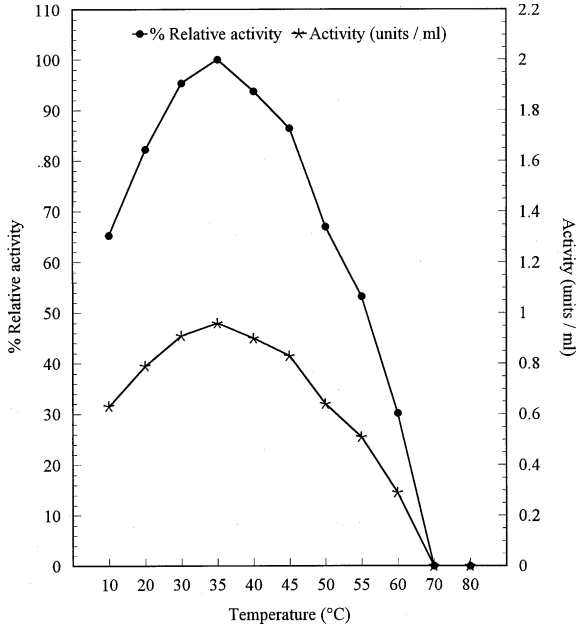

Fig. 6. Effect of temperature on the activity of the acidic protease of bolti.

3.2.4. Thermostability

Thermostability of the acidic protease is shown in Fig. 7. The acidic protease retained more than 50% of its activity after heating at 50 and 60°C for 30 min, while it lost 40.2% and 74.9% after heating at the same

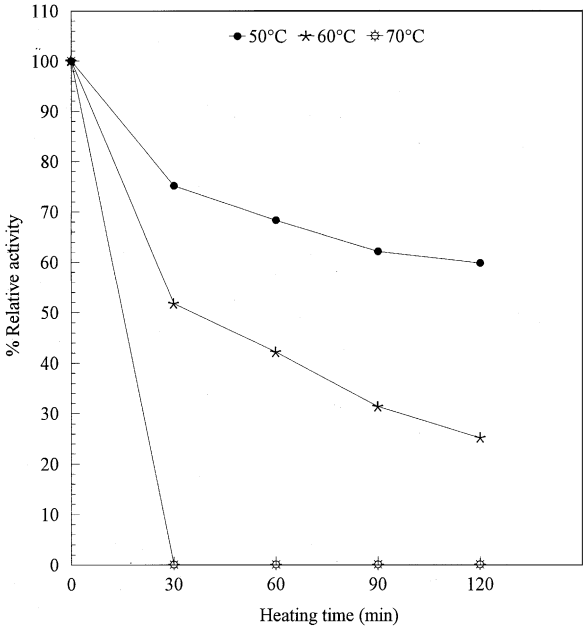

Fig. 7. Effect of heating time on the stability of the acidic protease of bolti.

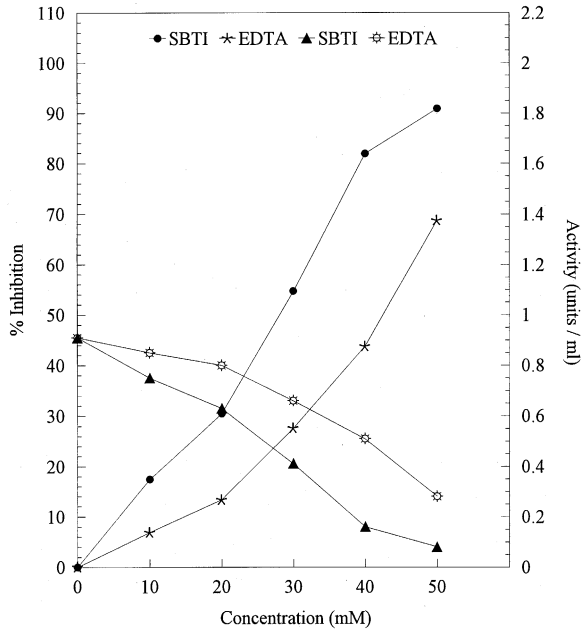

Fig. 8. Influence of some inhibitors on the activity of the acidic protease of bolti.

temperature for 120 min. The enzyme lost all its activity when heated at 70°C for 30 min. These results are in agreement with those reported by Dimes et al. (1994) and Garcia-Carreño and Haard (1993).

The previous data support the idea that proteolytic enzymes from bolti fish have unique thermal properties, being more susceptible to thermal inactivation than those of animals living at higher temperature (Osnes and Mohar, 1985).

3.2.5. Determination of K_m and V_{max}

A straight line for acidic protease was obtained when $^1/_V \times 10^{-1}$ was plotted as a function of $^1/_S \times 10^{-1}$. The vertical intercept is equal to $^1/v_{max}$ being 0.45 and the horizontal negative intercept was $-^1/K_m$ being 1.3. The K_m and V_{max} values of acidic protease were calculated as 0.77 mM and 2.22 mM/min, respectively. The catalysis efficiency (V_{max}/K_m) was 2.88. These results are higher than those reported by Ibrahim (1994) who reported that K_m and V_{max} were 0.180 and 0.044 for crude bolti acidic protease. However, they are lower than those obtained by Arunchalam and Haard (1985), who reported that K_m and V_{max} of Polar cod gastric protease were 1.33 and 31.00, respectively.

3.2.6. Enzyme inhibitors

High inhibition percentages of total acidic protease activity were obtained when the acidic protease was incubated with 50 mM of both soybean trypsin inhibitor or ethylenediaminetetraacetic acid (90.9% and 68.8%, respectively), while they were 17.4% and 6.9%, respectively, when 10 mM concentration was used (Fig. 8). Generally, these data agree well with those reported by Diaz-López et al. (1998) for the acidic protease. Moyano López, Martinez Diaz, Diaz Lopez & Alarcon Lopez (1999) reported that tilapia digestive proteases showed great sensitivity to the protease inhibitor present in defatted soybean since a high inhibition rate (nearly 40%) was reached, even with very low concentration. The in vitro information of inhibition of tilapia proteases does not properly reflect what occurs in vivo, where other factors could modify physiological response of fish (El-Sayed, Martinez, & Moyano, 2000).

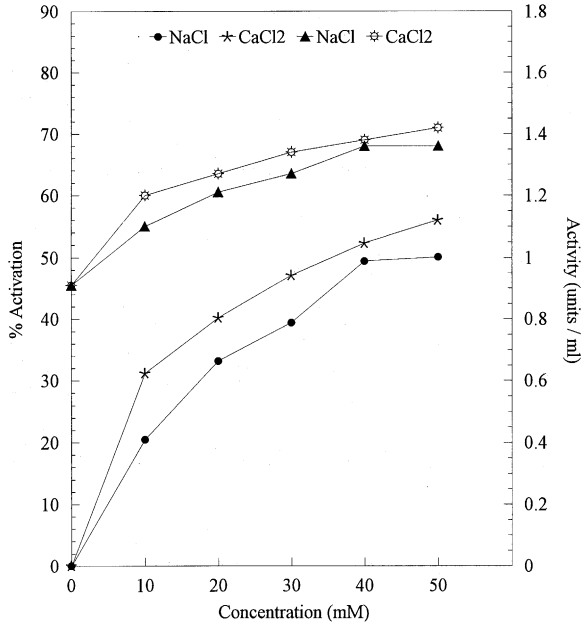

Fig. 9. Influence of some activators on the activity of the acidic protease of bolti.

3.2.7. Enzyme activators

The effects of various concentrations (10–50 mM) of NaCl and $CaCl_2$ on the activity of partiallly purified acidic protease are shown in Fig. 9. Presence of both NaCl and $CaCl_2$ (10 mM), in the reaction mixture, increased the partially purified acidic protease activity by 20.5% and 31.2%, respectively. The activation percentage was increased with increase of activator and reached 50.1% and 56.1% when 50 mM of both NaCl and $CaCl_2$ were used. These data are parallel to those reported by Raksakulthai and Haard (1999) for squid aminopeptidase. Such results are not quite in line with those of Squires, Haard, and Felthame (1986b) who reported that porcine pepsin showed slight inhibition at higher levels of NaCl, while cod protease was unaffected by the presence of NaCl. However, the protease activity of Green land Cod was dramatically increased when NaCl was used.

4. Conclusion

The viscera of Bolti fish contained considerable amounts of acidic proteases which can be used as different food processing aids, thereby contributing to reducing the waste disposal problem. The obtained enzyme had a molecular weight of about 31.00 kDa, pH optimum of 2.5, temperature optimum 35°C, K_m and V_{max} 0.77 and 2.22, respectively. The obtained crude acidic protease characteristics are close to those reported previously for acidic proteases of some marine fishes.

References

Anson, M. L., & Mirsky, A. E. (1932). The estimation of pepsin with hemoglobin. *Journal of General Physiology, 16*, 59–63.

Arunchalam, K., & Haard, N. F. (1985). Isolation and characterization of pepsin from Polar cod (*Boreogadus saida*). *Comparative Biochemistry and Physiology, 80B*(3), 467–473.

Chaplin, M. F., & Bucke, C. (1990). *Enzyme technology*. Cambridge, MA: Cambridge University Press.

Davis, B. J. (1964). Disc electrophoresis. 2-Method and application to human serum protein. *Annals of the New York Academy of Sciences, 121*, 404.

Dimes, L. E., Garcia-Carreño, F. L., & Haard, N. F. (1994). Estimation of protein digestibility. 3. Studies on the digestive enzymes from the pyloric caeca of rainbow trout and salmon. *Comparative Biochemistry and Physiology, 109A*, 349–360.

El-Sayed, A. M., Martínéz, I. N., & Moyano, F. J. (2000). Assessment of the effect of plant inhibitors on digestive proteases of *Nile Tilapia Nilotica* using in vitro assay. *Aquaculture International, 8*, 403–415.

Garcia-Carreño, F. (1991). Proteases in Food Technology. *Biotechnology Education, 2*, 150–153.

Garcia-Carreño, F. L., & Haard, N. F. (1993). Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. *Journal of Food Biochemistry, 17*, 97–113.

Godfrey, T., & Reichelt, J. (1983). *Industrial enzymology, the application of enzyme in industry*. London: Macmillian Puplisher Ltd.

Haard, N. F. (1986). Atlantic cod gastric protease characterization with casein and milk substrate and influence of Sepharose immobilization on salt activation, temperature characteristics and milk clotting reaction. *Journal of Food Science, 51*, 313–316.

Haard, N. F. (1992). A review of proteolytic enzymes from marine organisms and their application in the food industry. *Journal of Aquatic Food Product Technology, 1*(1).

Haard, N. F. (1998). Specialty enzymes from marine organisms. *Food Technology, 52*, 64–67.

Haard, N. F., & Simpson, B. K. (1994). In A. M. Martin (Ed.), *Fisheries processing: Biotechnological application* (pp. 132–153). London: Champan & Hall.

Haard, N. F., Feltham, L. W., Helbig, N., & Squires, J. (1982). Modification of protein with proteolytic enzymes from the marine environment. *Advances in Chemistry, 198*, 223–244.

Ibrahim, M. H. (1994). Utilization of some food processing wastes. PhD Thesis, Faculty of Agriculture Ain Shams University, Cairo, Egypt.

Kemény, A., Varga, J., Kárpáti, M., Láztity, R., Vadon, E., & Korányi, P. (1989). Separation of cereal and vegetable seed protein by gel electrophoresis. *Journal of Planar Chromatography, 2*, 368–380.

Lowry, O. H., Resebrough, N. J., Far, A. L., & Randell, R. J. (1951). Protein measurement with Folin Phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Lineweaver, H., & Burke, D. (1934). Determination of enzyme dissociation constants. *Journal of American Chemical Society, 56*, 658–666.

Lopez-Liorca, L. V. (1990). Purification and properties of extra cellular proteases produced by the nematophagous fungus *Verticillium suchlospovium*. *Canadian Journal of Microbiology, 39*, 530–537.

Moyano López, F. J., Martinez Diaz, I., Diaz López, M., & Alarcón López, F. J. (1999). Inhibition of digestive proteases by vegetable meals in three fish species seabream (*Saparus aurata*), tilapia (*Oreochromis niloticus*) and African sole (*Solea senegalensis*). *Comparative Biochemistry and Physiology, B122*, 327–332.

Osnes, K. K., & Mohar, V. (1985). On the purification and characterization of three anionic, serine type peptide hydrolases from Atlantic krill. *Comparative Biochemistry and Physiology, 82B*, 607–619.

Raksakulthai, R., & Haard, N. F. (1999). Purification and characterization of amino peptidase fractions from squid (*Illex illecebrosus*) hepatopanecreas. *Journal of Food Biochemistry, 23*, 123–144.

Simpson, B. K., Simpson, M. V., & Haard, N. F. (1990). Properties of trypsin from the pyloric caeca of Atlantic Cod (*Gadus morhua*). *Journal of Food Science, 55*, 959–971.

Squires, E. J., Haard, N. F., & Felthame, L. W. (1986a). Gastric proteases of the Greenland Cod *Gadus ogac*. I. Isolation and kinetic properties. *Biochemistry and Cell Biology, 64*, 205–214.

Squires, E. J., Haard, N. F., & Felthame, L. W. (1986b). Gastric proteases of the Greenland Cod *Gadus ogac*. II. Structural properties. *Biochemistry and Cell Biology, 64*, 215–222.

Yamamoto, A. (1975). In G. Reed (Ed.), *Enzymes in food processing* (p. 123). New York: Academic Press.