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Purification and Characterization of Alkaline Proteases from the Viscera of Mackerel (*Scumber scumbrus*) and Macaroni Fish (*Saurus tumbil*)

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AN ALKALINE protease was extracted from acetone powder prepared from the viscera of Mackerel (*Scumber scumbrus*) and Macaroni (*Saurus tumbil*) fish, precipitated from the extract by 40-60% ammonium sulfate, dialyzed and purified by gel exclusion chromatography. The optimal pH and optimal temperature of both enzymes were 8 and 50°C, respectively (using BAPNA as a substrate). Remarkable stability was observed at pH 6, where more than 75 % of the activity of both enzymes remained after 60 min while they retained more than 60% when incubated for the same period at pH 5.0. Furthermore, both enzymes retained more than 50% of their activity after heating at 50°C for 90 min, while, they were completely inactivated when heated at 60°C for 150 min. Gel exclusion chromatography increased the purification to 15.12 and 19.67-fold for Mackerel and Macaroni enzyme extracts, respectively. Only a single protein band with an R_f value corresponding to a molecular weight of 20,000 Da was observed with the enzyme extracted from Macaroni while two bands with R_f values close to 20,000 and 28,000 Da were noticed in the Mackerel extract when sodium dodecyl sulphate polyacrylamid gel electrophoresis (SDS-PAGE) was used for molecular weight determination.

Keywords: Mackerel fish, Macaroni fish, Viscera, Alkaline protease, Extraction, Purification, Kinetic reaction.

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. However, fishery by-products are typically feeds and fertilizers that have a low dollar value. There is growing interest in obtaining higher value biochemical and pharmaceuticals from fishery wastes, notably enzymes (Haard, 1998). In recent years, proteolytic enzymes from marine organisms have been used as process aids. Other possible uses of enzymes from marine animals in the food industry are based on the unique properties of these enzymes, *i.e.*,ease of heat denaturation, high molecular

activity at low temperature and ability to catalyze hydrolysis of native proteins (Haard, 1992 and Dimes *et al.*, 1994).

In Egypt, about half of the fish offal generated is used as fish meal, while the remainder represents a disposal and pollution problem. The utilization of the aforementioned materials as a source of industrial enzyme would serve as a means of minimizing the waste disposal problem of the fishing industry, as well as recover valuable materials that could be used as food processing aids (Ibrahim, 1994 and El-Beltagy *et al.*, 2004). Recently, a number of studies designed to characterize digestive enzymes of aquatic organisms have been performed (Alarcon *et al.*, 1998; El-Beltagy *et al.*, 2004 and El-Beltagy *et al.*, 2005). Based on the aforementioned points of view, this research is carried out as a contribution to the utilization of fish by-products as a source of alkaline proteases having enhanced value.

Material and Methods

Materials

Fish viscera samples

Mackerel (*Scumber scumbrus*) and Macaroni (*Saurus tumbil*) were purchased from a local market at Giza, Egypt and the viscera (300gm) were removed by hand and then stored in sealed plastic bags at – 20°C.

Reagents

N_{α}-benzoyl-$_{DL}$-arginine-$_{p}$-nitroanilide (BAPNA), Bovine serum albumin, trichloroacetic acid, glycerol, β-mercaptoethanol, tris (hydroxymethylamino-methane), Commassie 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. A mixture protein standards was purchased from Bio-Rad, Mississauga, Ontario, Canada. Sephadex G-100 was purchased from Pharmacia Fin Chemical Co. Uppsala, Sweden.

Methods

Preparation of crude alkaline protease

Digestive tracts (viscera) were partially thawed in the refrigerator at 4°C, defatted by homogenization with 8 volumes of cold acetone (4°C) in a homogenizer (Edmund Bühler, Tühler,Tübingen, Typ. Hov Nr. 52). The homogenate was filtered through four layers of cheese cloth and the acetone insoluble material was washed several times with cold acetone and finally once with ether and then dried at room temperature 25 ± 2°C overnight (acetone powder). The powder was kept in a brown bottle and stored at – 20°C for further analysis. The dry acetone powder was mixed with distilled water (1: 20, w/v) for 1hr using a mechanical shaker (Haake SWB 20, Germany). The resulted supernatant was referred to as crude alkaline protease (Hjelmeland and Raa, 1980).

Ammonium sulfate precipitation

From the preliminary studies, alkaline proteases were collected from their crude supernatant by precipitation with 40 – 60% saturated ammonium sulfate. After agitation for 30 min at room temperature at 25±2°C, the resulting precipitates were allowed to settle for 24 hr at 4°C. The supernatants were discarded and the precipitates dissolved in 0.05 M Tris HCl buffer, pH 7.8.

Dialysis

The resulted fraction was dialyzed against 0.05 M Tris HCl buffer of pH 7.8 at 4°C for 24 hr, the buffer was changed twice and the dialyzed enzyme was kept in brown bottles (partially purified alkaline protease) and stored at –20°C for further purification.

Gel filtration

The dialyzed enzyme was fractionated on a Sephadex G-100 column of 1 × 50cm. The column was equilibrated and eluted with 0.05 M Tris HCl buffer pH 7.8. Elution was carried out at a flow rate of 20 ml / hr and 3 ml fractions were collected manually. Absorbance and enzyme activity were measured in all fractions at 280 nm in cuvets of 1cm path length using a spectrophotometer (Beckman Du 7400 Spectrophotometer, USA). The major active fractions were collected and stored at – 20°C for further analysis.

Determination of the molecular weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of the purified enzymes 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) vertical gel tubes containing 0.15% SDS and 0.375 M TRIS were prepared. The running buffer contained 3.03g tris, 14.42g glycine and 1g SDS per litter. Then, 100 µl of samples were applied to the gel surface and fractionated for 3 hr at 300 voltages under refrigeration. The gel was stained with 25mg 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 calibration proteins were used as molecular weight markers. The relative mobilities were calculated and plotted against the logarithm of molecular weight for determining the molecular weight of proteins.

Enzyme assay

Enzyme activity was determined using ***N_α-benzoyl-$_{DL}$-arginine-$_p$-nitroanilide*** (BAPNA) as substrate (Erlanger *et al.*, 1961). BABNA (43.5mg) was desolved in 1ml dimethylsulfoxide, and then brought to 100ml with 0.05 M Tris-Hcl buffer (pH 7.8) containing 0.02 M $CaCl_2$. One BABNA unit is defined as (ΔA 410nm/min x 1000 x 3) / 8800 where 8800 is the extinction coefficient of $_p$-nitroanilide.

Protein content

Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

pH optima and stability

The optimum pH was determined by preparing the BAPNA substrate in the following buffer solutions (0.05 M citrate phosphate buffer of pH 3.0 – 6.0 and 0.05 M Tris–HCl buffer of pH 7.0 – 10.0). The influence of pH on the stability of the protease was determined by pre-incubating the enzyme in citrate phosphate buffer at pH 5.0 and 6.0 for 30, 60, 90, 120 and 150 min, at room temperature (25 $\pm$ 1°C), readjusting to pH 7.0 and determining the remaining activity.

Temperature optima and thermostability

The influence of temperature on the activity of the alkaline protease was determined at various temperature intervals (10 to 80°C). For stability the enzyme solution was incubated at 50 and 60°C for 30, 60, 90, 120 and 150 min, then cooled rapidly in an ice bath for 5 min and the residual activity assayed.

Results and Discussion

Purification of enzyme crude extract

Table 1 summarizes the specific activity and fold-purification of alkaline proteases extracted from Mackerel and Macaroni viscera. The crude extract of Mackerel protease had a higher specific activity (0.08U/mg protein)than that of Macaroni (0.06U/mg protein). Purification by 40-60% ammonium sulfate increased the specific activity of both enzymes to 0.46 and 0.42 U/mg protein, respectively. Dialysis slightly increased the specific activity to 0.47 and 0.43U/mg protein resulting in 5.87 and 7.17-fold purification for Mackerel and Macaroni enzyme extracts, respectively. Gel filtration highly increased the purification to 15.12 and 19.67-fold for Mackerel and Macaroni enzyme extracts, respectively. One peak has been detected from gel filtrated Macaroni extract (sharp peak) and Mackerel (broader peak). Also, the specific activity of both enzymes was increased to 1.21 and 1.18 U/mg proteins, respectively by gel filtration. Raksakulthai & Haard (1999) stated that the dialysis of amino peptidase crude extract (from squid hepatopanerease) increased the purification from 2.08 to 4.6-fold after purification by ammonium sulfate. Gel chromatography increased the purification of alkaline proteases extracted from Bolti fish to 20.6-fold (El-Beltagy *et al.*, 2005).

TABLE 1. Purification steps of alkaline protease isolated from the viscera of Mackerel and Macaroni fish.

Purification steps	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units / mg protein)	Recovery (%)	Fold purification
Mackerel Crude extract	500	1735	21240	0.08	100	1.00
Ammonium sulfate (40 – 60%)	50	1122	2420	0.46	64.67	5.75
Dialysis	50	1086	2316	0.47	62.59	5.87
Gel filtration	17	301	249	1.21	17.35	15.12
Macaroni Crude extract	500	1652	26870	0.06	100	1.00
Ammonium sulfate (40 – 60%)	50	906	2170	0.42	54.84	7.00
Dialysis	50	840	1961	0.43	50.85	7.17
Gel filtration	15	252	213	1.18	15.25	19.67

$$\text{Specific activity} = \frac{\text{Total activity (units)}}{\text{Total protein (mg)}}$$

$$\text{\% Recovery} = \frac{\text{Total activity}}{\text{Total activity of crude extract}} \times 100$$

$$\text{Fold -purification} = \frac{\text{Specific activity}}{\text{Specific activity}}$$

Temperature optima and thermostability

The optimum temperature of both alkaline proteases isolated from Mackerel and Macaroni viscera had their highest activity at 50°C (when assayed with BABNA as a substrate), while no activity was detected proteases from both species when assayed at 80°C (Fig. 1). The activity of both alkaline proteases increasing up to 50°C. The alkaline protease extracted from Macaroni viscera had slightly higher activity than that from Mackerel at certain temperatures. Both enzymes retained more than 89% of their activity when

heated at 50°C for 30 min while, they retained less than half of their activity when heated for 120 min at the same temperature (Fig. 2). Both enzymes retained more than 60% of their activity when heated for 30 min at 60°C. Moreover, they lost more than 50% of their activity when heated for 60 min at the same temperature and were completely destroyed when heated at the same temperature for 150 min (Fig. 3). These results are similar to those obtained by Alarćon *et al.* (1998) which reported that the optimum temperature of the alkaline protease from seabream (*Sparus aurata*) and common dentex (*Dentex dentex*) ranged from 50-55°C, and that the seabream trypsin-like enzyme remained completely active at 50°C.

pH optima and pH stability

The activity of both alkaline protease increased with increasing pH and reached its maximum at pH 8.0 and then decreased with further increase of pH (Fig. 4). Both enzymes had a high relative activity (more than 76%) within a pH range 6.0 – 9.0, while they lost more than 60% of their activity at pH less than 5.0. Simpson *et al.* (1990) stated that Atlantic cod trypsin was most active at pH 7.5 when N_α *benzoyl-* $_{DL}$*-arginine-* $_p$*-nitroanilide* (BAPNA) was used as a substrate and that the enzyme had the highest activity within the pH range of 7.0 – 10.0.

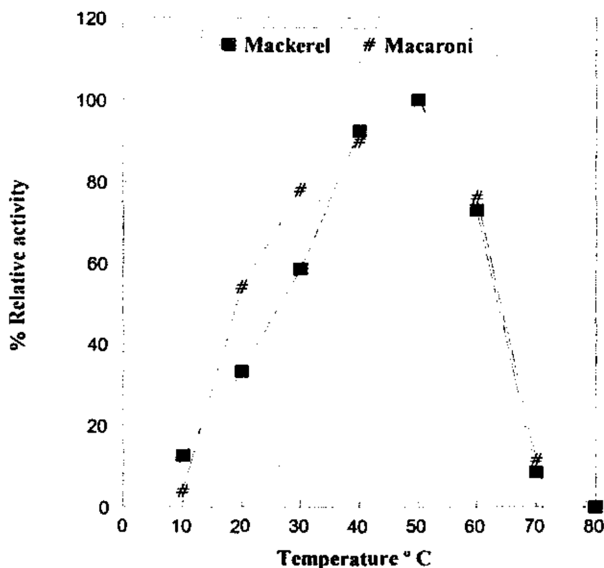

Fig. 1. Effect of temperature on alkaline protease activity of Mackerel and Macaroni viscera.

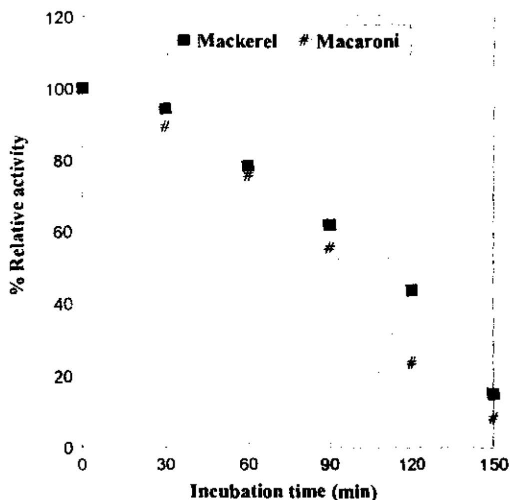

Fig. 2. Thermostability (50 °C) of alkaline proteases extracted from Mackerel and Macaroni viscera.

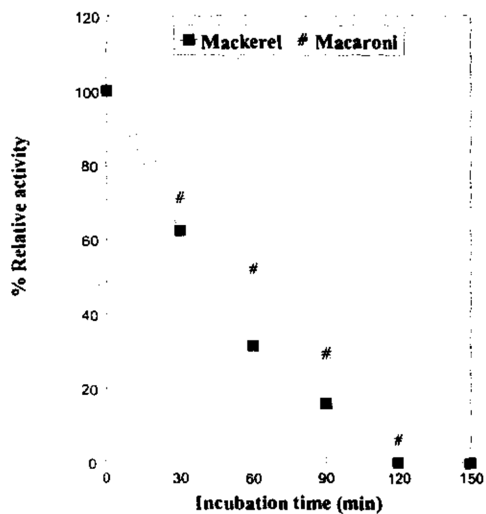

Fig. 3 . Thermostability (60 °C) of alkaline proteases extracted from Mackerel and Macaroni viscera.

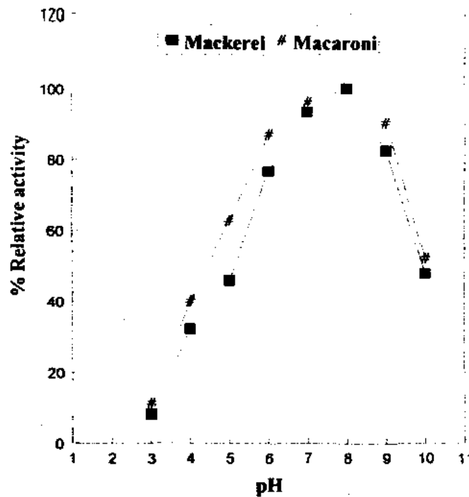

Fig. 4. Effect of pH on the activity of alkaline proteases extracted from Mackerel and Macaroni viscera.

Figure 5 shows the pH stability (at pH 5) of alkaline protease extracted from Mackerel and Macaroni viscera. A remarkable decrease in the residual activity of both enzymes extract was observed. Both enzymes retained more than 60% of their activity when incubated for 60min while, they lost more than 80% of their activity when incubated for 150min. The alkaline enzyme extracted from Mackerel showed a higher pH stability than that from Macaroni and they both lost less than 50 % of their activity when incubated for 90 min at pH 6.0 but, they retained 23.47 and 17.73%, respectively when incubated for 150 min (Fig. 6).

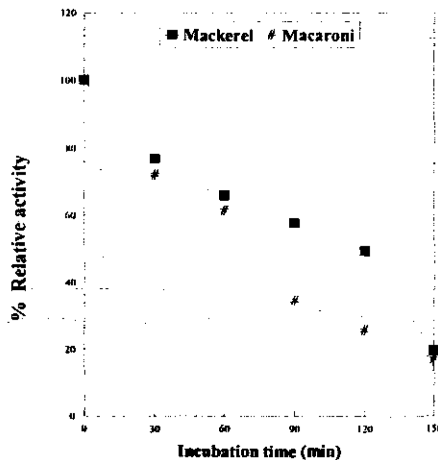

Fig. 5. pH stability (pH 5) of alkaline proteases extracted from Mackerel and Macaroni viscera.

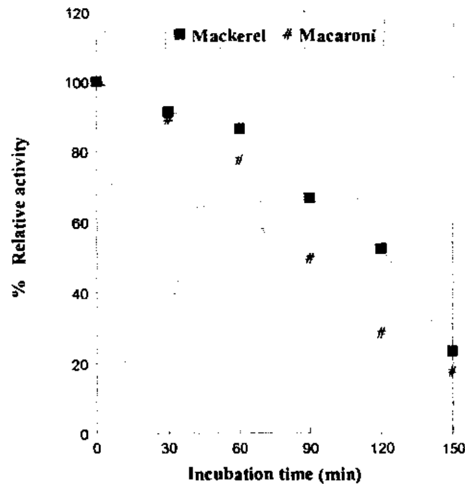

Fig. 6 . pH stability (pH 6) of the alkaline proteases extracted from Mackerel and Macaroni viscera.

Determination of enzymes molecular weight

Molecular weight of alkaline proteases eluted from column chromatography was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Only a single protein band with R_f corresponding to a molecular weight of 20,000 Da was observed in the enzyme extracted from Macaroni, while two bands with R_f close to 20,000 and 28,000 Da were noticed in Mackerel (Fig. 7). Simpson & Haard (1987) reported that trypsin obtained by affinity chromatography migrated as a single band on SDS gel corresponding to a molecular weight of 24,000 Da. Simpson *et al.* (1990) reported that the molecular weight of Atlantic Cod pyloric caeca was 23,447 Da calculated on basis of amino acid composition. Similar results for seabream were mentioned by Alacron *et al.* (1998).

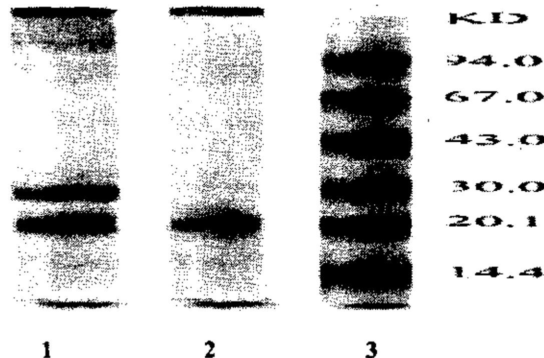

Fig. 7. SDS-PAGE pattern of pure Mackerel and Macaroni alkaline protease.

1. **Pure Mackerel alkaline protease**
2. **Pure Macaroni alkaline protease.**
3. **Standard proteins.**

Conclusion

The viscera of both Mackerel and Macaroni fish contained a considerable amount of alkaline protease which has the potential for use as aids in various food and other commercial processes thereby increasing value of such waste. The extracted enzymes had a molecular weight of about 20.00 KDa for Macaroni, while two protein fractions (20.00 and 28.00 KDa) were detected in extracted Mackerel viscera. Both extracted enzymes had pH optima of 8.0 and temperature optima of 40°C. The alkaline enzyme extracted from Mackerel showed exhibited somewhat greater stability at a pH 5.0 than that from Macaroni.

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تنقية و خصائص إنزيمات البروتيز القاعدية المستخلصة من أحشاء أسماك الماكريل و المكرونة

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تم استخلاص إنزيمات البروتيز القاعدية من مسحوق الأسيتون لأحشاء كل من اسماك المكرونة و الماكريل حيث تم ترسيبها باستخدام كبريتات الأمونيوم ٤٠ – ٦٠ ٪ ثم إجريت عملية الديلزة و الترشيح بالجيلى.

كانت درجة الـ pH المثلى و درجة الحرارة المثلى لكلا الإنزيمين ٨ و ٥٠ م° على التوالى (باستخدام BAPNA كمادة تفاعل). تم تسجيل ثبات ملحوظ على درجة ال pH ٦ حيث احتفظ كلا الإنزيمين بأكثر من ٧٥ ٪ من نشاطهم بعد مرور ٦٠ دقيقة ، بينما عند تحضينهما لنفس المدة على درجة pH ٥ احتفظت الإنزيمات بحوالى ٦٠٪ من نشاطهما. بالإضافة إلى ذلك إحتفظ كلا الإنزيمين بأكثر من ٥٠٪ من نشاطهما بعد إجراء عملية التسخين على ٥٠م° و لمدة ٩٠ دقيقة، بينما تم تثبيط هذه الإنزيمات بالكامل بالتسخين على درجة حراره ٦٠ م° و لمدة ١٥٠ دقيقة .

أدت عملية الترشيح الجيلى Gel filtration إلى زيادة درجة التنقية إلى ١٥,١٢ و ١٩,٦٧ لكل من مستخلصات الإنزيم للماكريل و المكرونة على التوالى.

تم ملاحظة باند واحد فقط من البروتين وزنة الجزيئى ٢٠٠٠٠ دالتون فى المستخلص الإنزيمى الناتج من المكرونة بينما وجد باندين وزنهما الجزيئى ٢٠٠٠٠ و ٢٨٠٠٠ دالتون فى المستخلص الإنزيمى الناتج من الماكريل وذلك باستخدام نموذج الهجرة الكهربية (الإلكتروفوريسيس) على جيل البولى أكريلميد لتقدير الوزن الجزيئى.