Bull. Fac. Fish. Hokkaido Univ.
46(3), 63-70. 1995.

Proteinase from the Liver of Neon Flying Squid: Purification and properties

SUKARNO[1], Koretaro TAKAHASHI[1], Mutsuo HATANO[1]
and Yasunori SAKURAI[2]

Abstract

A proteinase from the liver of neon flying squid (*Ommastrephes bartramii*) has been partially purified by means of organic solvent extraction and successive chromatographies, i.e., anion exchange chromatography on DEAE-Cellulofine A-500 and gel permeation chromatography on Sephadex G-100. The molecular weight of the enzyme estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 24,000. The enzyme's optimum pH was approximately 5.0, and the enzyme was relatively stable over a pH range of 4.0 to 7.0. The optimum temperature was approximately 37°C, and the enzyme was tolerably stable up to 37°C. The enzyme was significantly activated by 2-mercaptoethanol, dithiothreitol, cysteine, glutathione, K^+, NH_4^+, Li^+, Na^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, and slightly activated by sodium azide and ethylenediaminetetraacetic acid. In contrast, the enzyme was significantly inhibited by iodoacetamide, iodoacetic acid, soybean trypsin inhibitor, N-ethylmaleimide, *p*-hydroxymercuribenzoate sodium salt, *p*-chloromercuribenzoate, 5-5′-dithiobis-(2-nitrobenzoic acid), hydroquinone, *o*-phenanthroline, Fe^{2+}, Hg^+, Ag^+, and slightly inhibited by diisoprophylfluorophosphate and phenylmethylsulfonyl fluoride. These results show that the enzyme is a cysteine proteinase.

Key Words: Hydrolytic Enzyme, Liver, *Ommastrephes bartramii*, Proteinase, Squid

Introduction

Proteinase is widely distributed in animals, plants, and microorganisms. Numerous published reports on the purification and application of this enzyme have indicated that the properties of purified proteinase, e.g., substrate specificity, thermostability, pH stability, activator requirements, etc., vary widely. In contrast to the wealth of information about proteinase from other biological sources, there have been no reports on proteinase from the neon flying squid (*Ommastrephes bartramii*).

The objective of this study was to investigate the possibility of using the liver of neon flying squid, commonly discarded during processing, as a potential source for industrially valuable proteinase with special characteristics. In addition, by using an organ that is usually discarded as a source of enzyme, it is expected to reduce environmental pollution and increase the economical value of the waste. Results of purification and some properties of this proteinase are described.

[1] *Laboratory of Food Biochemistry, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan* (北海道大学水産学部食品生化学講座)
[2] *Laboratory of Marine Ecology, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan* (北海道大学水産学部資源生態学講座)

Materials and Methods

Preparation of the crude enzyme

Neon flying squids were caught near the Midway Islands in July, 1993. From 30 squids collected, the mean body length, body weight and liver weight were 40.1 cm, 987.0 g and 193.8 g, respectively. Crude enzyme was prepared by extraction of the liver using organic solvents. The method used was as follows: fifty grams of squid liver was mixed in 10 volumes of cold acetone for 30 min, homogenized for 5 min, then centrifuged at $10,000 \times g$ for 30 min. The precipitate formed was collected, treated again with cold acetone and then recentrifuged. The same procedure was repeated with both ethyl acetate and diethyl ether. The final precipitate was vaccum-dried to obtain the organic solvent powder, which was used for further studies.

Chromatographic purification of proteinase

The crude enzyme obtained from organic solvent extraction was dialyzed against 0.05 M phosphate buffer, pH 7.5, for 24h at 4°C, and then loaded on a DEAE-Cellulofine A-500 anion exchange chromatography column (2.5×40 cm) (Seikagaku Kogyo Co., Ltd., Tokyo, Japan). Proteins were eluted with 0.05 M phosphate buffer, pH 7.5, containing 0.3 M NaCl and 0.02% benzalkonium salt, at 4°C. Fractions of 4 ml were collected by a fraction collector at a flow rate of 8 ml/cm^{2}h. The effluent was monitored at 280 nm using a Hitachi U-2000 spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan).

The active fraction concentrated with a water-absorbent polymer (Sanyo Kasei Co., Ltd., Tokyo, Japan) was then applied to a Sephadex G-100 gel permeation chromatography column (2.0×85 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden). Proteins were eluted with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02% sodium azide (NaN_3) at 4°C, and the flow rate was 2 ml/cm^{2}h. The effluent was monitored at 280 nm. Before treatment with vacuum-drying, each fraction from chromatographic purification was treated with a water-absorbent polymer for 24h. Then, they were subjected to an enzyme activity test. The protein content of each fraction was determined using the method described by Lowry et al. (1951). Vacuum-drying was carried out using a Tokyo Rikakikai FD-5 freeze-drier.

Molecular weight estimation

The molecular weight of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a modified Laemmli discontinuous buffer system (Anonymous, 1986).

Assay of proteolytic activity

Proteolytic activity was determined according to the method of Hagihara et al. (1958), a modification of the methods of Anson (1938) and Kunitz (1947). In brief, 1 ml of sample was added to 1 ml of 2% casein solution in 0.2 M phosphate buffer, pH 5.0, and incubated at 37°C for 10 min. After cooling, 2 ml of Hagihara's reagent (consisting of 0.11 M CCl_3COOH, 0.22 M CH_3COONa and 0.33 M CH_3COOH) was added to the reaction mixture and incubated at 37°C for 20 min. After filtration with Toyo filter paper No. 5A (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), 5 ml of 0.5

M Na_2CO_3 and 1 ml of 0.33 M phenol reagent were added to 1 ml of the filtrate and incubated at 37°C for 20 min. The optical density of the reaction mixture was measured at 660 nm, and the amount of tyrosine (μg/ml) was calculated from a calibration curve using tyrosine as a standard. The total activity was then calculated from the following formula: total activity (total amount of tyrosine, μg) = amount of tyrosine (μg/ml) $\times$ vol (ml). One unit of proteolytic activity (PU) was defined as the amount of enzyme that gave the optical density at 660 nm equivalent to 1.0 μg of tyrosine in 1 min.

Measurements of optimum pH and pH stability

The optimum pH of the enzyme was determined at various pH levels from 4.0 to 10.0, using 0.2 M phosphate buffer for pH levels of 4.0 to 8.0, and 0.2 M Tris-HCl buffer for pH levels of 7.0 to 10.0. Assays were measured spectrophotometrically as described above. The relative activity was based on the highest activity of the enzyme at a certain pH (as 100%). To examine pH stability, the pH levels of the enzyme solutions were adjusted with those buffers and kept at 4°C overnight. After the treatment, the enzyme solutions were diluted with 0.2 M phosphate buffer (at optimum pH). The residual activity was expressed as a percentage of non-treated proteinase activity.

Measurements of optimum temperature and thermal stability

The optimum temperature of the enzyme was determined at various temperatures, i.e., 4, 15, 25, 37, 45, 50, 55, 60 and 70°C. Assays were measured spectrophotometrically as described above. The relative activity was based on the highest activity of the enzyme at a certain temperature (as 100%). The thermal stability was investigated by determining the residual activity after leaving the enzyme at 4, 20, 37 and 42°C for 3 days, and at 60 and 100°C for 60 min. The residual activity was expressed as a percentage of non-treated proteinase activity.

Effects of some chemicals on the activity

The partially purified proteinase was incubated according to the assay method described above in the presence of some chemicals, e.g., inhibitors, reductants, metal ions, etc. The chemicals were: 0.1 mM diisoprophylfluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF), iodoacetamide, iodoacetic acid (IAA), 5-5′-dithiobis-(2-nitrobenzoic acid) (DNTB), *p*-chloromercuribenzoate (PCMB), *p*-hydroxymercuribenzoate (PHMB), N-ethylmaleimide (NEM) and soybean trypsin inhibitor (STI); and 1 mM 2-mercaptoethanol (2-Me), glutathione (GSH), cysteine, dithiothreitol (DTT), *o*-phenanthroline, hydroquinone, ethylenediaminetetraacetic acid (EDTA), sodium azide (NaN_3), Ag^+, Hg^+, Fe^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Na^+, Li^+, NH_4^+ and K^+. The relative activity was expressed as a percentage ratio of the specific activity of samples with various chemicals to a control with no chemical.

Results and Discussion

Chromatographies

Using DEAE-Cellulofine A-500 anion exchange chromatography, several peaks were detected and collected as three major fractions (data not shown). These main

fractions were treated with a water-absorbent polymer for 24h and then lyophilized. Among these, fraction I showed protease activity. The active fraction was then loaded on a Sephadex G-100 gel permeation chromatography column (2.0×85 cm). Two peaks were detected and collected as two fractions. These fractions were treated with a water-absorbent polymer for 24h and then lyophilized. Fraction II showed protease activity (data not shown). The lyophilized product was stored at 4°C, and used for study of properties of this enzyme.

Molecular weight

The molecular weight of the partially purified enzyme was estimated by SDS-PAGE. The marker proteins (molecular weight) used were ribonuclease A (13,700), chymotrypsinogen (25,000), ovalbumin (43,000) and bovine serum albumin (67,000). There were more than one band of stained protein, and the molecular weight of the main band was calculated to be approximately 24,000 from a semilogarithmic plot of molecular weight against mobility (data not shown).

Effects of pH and temperature on the activity

The enzyme showed the highest activity at approximately pH 5.0 (Fig. 1). The enzyme was relatively stable over a pH range of 4.0 to 7.0 (Fig. 2). The optimum temperature of the enzyme reaction was approximately 37°C (Fig. 3). The enzyme was tolerably stable up to 37°C (Fig. 4).

Effects of some chemicals on the activity

Table 1 shows the effect of various cations on the enzyme activity. Addition

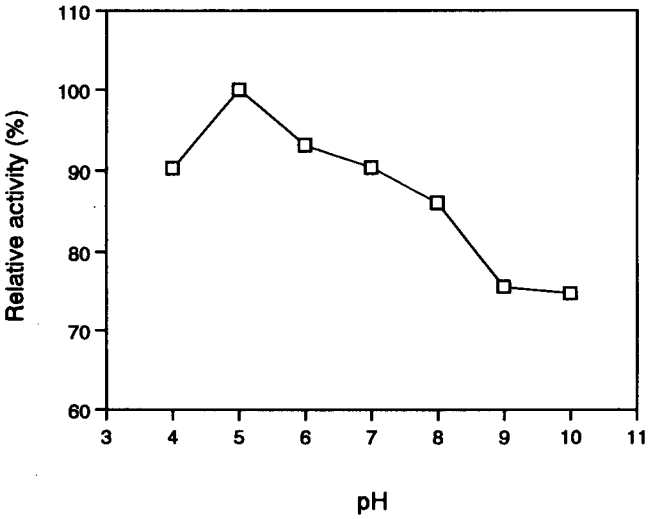

Fig. 1. Effect of pH on the proteinase activity.
The optimum pH of the enzyme was determined in the pH range of 4.0 to 10.0, using 0.2 M phosphate buffer for pH levels of 4.0 to 8.0, and 0.2 M Tris-HCl buffer for pH levels of 7.0 to 10.0. Assays were measured spectrophotometrically as described in the Materials and Methods. The relative activity was based on the highest activity of the enzyme at a certain pH (as 100%).

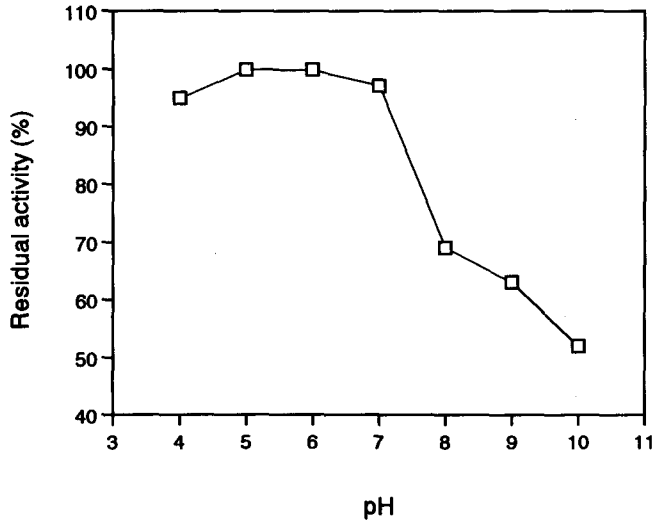

Fig. 2. pH stability of the proteinase examined.
To examine pH stability, the pH levels of the enzyme solutions were adjusted to various pH levels from 4.0 to 10.0, using 0.2 M phosphase buffer for pH levels of 4.0 to 8.0, and 0.2 M Tris-HCl buffer for pH levels of 7.0 to 10.0, and kept at 4°C overnight. After the treatment, the enzyme solutions were diluted with 0.2 M phosphate buffer (at optimum pH). The residual activity was expressed as a percentage of non-treated proteinase activity.

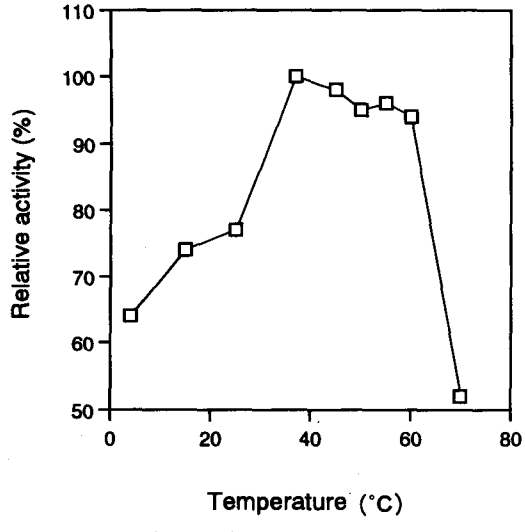

Fig. 3. Effect of temperature on the proteinase activity.
The enzyme activity was measured at various temperatures, i.e., 4. 15, 25, 37, 45, 50, 55, 60 and 70°C. Assays were measured spectrophotometrically as described in the Materials and Methods. The relative activity was based on the highest activity of the enzyme at a certain temperature (as 100%).

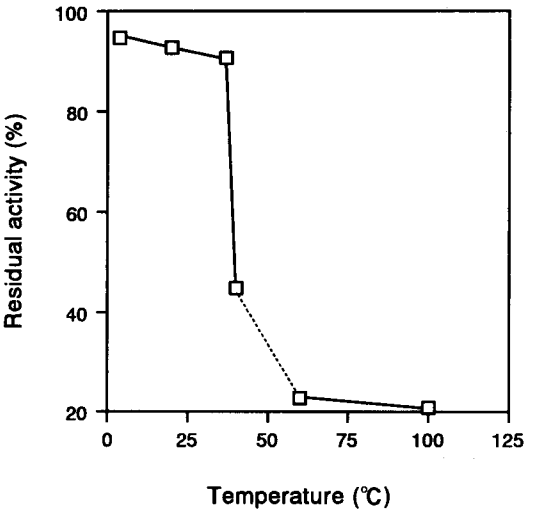

Fig. 4. Temperature stability of the proteinase examined.
The thermal stability was investigated by determining the residual activity after leaving the enzyme at 4, 20, 37 and 42°C for 3 days, and at 60 and 100°C for 60 min. The residual activity was expressed as a percentage of non-treated proteinase activity.

Table 1. Effect of metal ions on the proteinase activity.*

Metal ion (1 mM)	Relative activity (%)
None	100
Ag^{+}	75
Hg^{+}	54
Fe^{2+}	77
Zn^{2+}	153
Ba^{2+}	167
Mg^{2+}	144
Ca^{2+}	140
Na^{+}	157
Li^{+}	132
NH_4^{+}	155
K^{+}	162

* The enzume was incubated according to the assay method described in the Materials and Methods in the presence of each metal ion at a concentration of 1 mM. The relative activity was expressed as a percentage ratio of the specific activity of samples with various metal ions to a control with no metal ion.

of Zn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, Li^{+}, NH_4^{+} and K^{+} ions increased the initial activity by about 30 to 70%. An activating effect of Ca^{2+} was also reported for proteinase from *Streptococcus lactis* NCDO 763 (Monnet et al., 1987) and *S. cremoris* AC 1 (Geis et al., 1985). According to Exterkate and De Veer (1987), some cations have a

Table 2. Effect of various inhibitors on the ptoteinase activity.*

Inhibitor (0.1 mM)	Relative activity (%)
None	100
Diisoprophylfluorophosphate	90
Phenylmethylsulfonyl fluoride	95
Iodoacetamide	72
Iodoacetic acid	51
5-5′-Dithiobis-(2-nitrobenzoic acid)	72
p-Chloromercuribenzoate	67
p-Hydroxymercuribenzoate	32
N-ethylmaleimide	67
Soybean trypsin inhibitor	58

* The enzyme was incubated according to the assay method described in the Materials and Methods in the presence of each inhibitor at a concentration of 0.1 mM. The relative activity was expressed as a percentage ratio of the specific activity of samples with various inhibitors to a control with no inhibitor.

Table 3. Effects of reductants and chelating agent on the proteinase activity.*

Chemical (1 mM)	Relative activity (%)
None	100
2-Mercaptoethanol	130
Glutathione	131
Cysteine	134
Dithiothreitol	152
o-Phenanthroline	24
Hydroquinone	18
Sodium azide	114
Ethylenediaminetetraacetic acid	117

* The enzyme was incubated according to the assay method described in the Materials and Methods in the presence of these reductants and a chelating agent, with each at a concentration of 1 mM. The relative activity was expressed as a percentage ratio of the specific activity of samples with various reductants and a chelating agent to a control with no reductant or chelating agent.

structural function and stabilize the enzyme molecules in an active configuration. Meanwhile, Fe^{2+}, Ag^{+} and Hg^{+} ions reduced the initial activity of the enzyme by 23, 25 and 46%, respectively. This may be due to precipitation of the substrate by these ions, as indicated by Exterkate and De Veer (1987).

The effects of different inhibitors and reducing agents are shown in Tables 2 and 3. Proteinase activity was inhibited by addition of 0.1 mM iodoacetamide, IAA, DNTB, PHMB, NEM and STI. Here, iodoacetamide, IAA and NEM acted as alkylating agents. In addition, the enzyme was slightly inhibited by the presence of DIFP and PMSF, typical serine proteinase inhibitors. Thiol proteinase inhibitor, PCMB, had an inhibitory effect, reducing the initial activity by 33%.

Reducing agents, such as 2-Me, GSH, DTT, and cysteine, which stimulates thiol proteinase activity, caused an increase in the enzyme activity. EDTA also activated the enzyme. Altogether, our results indicate that the proteinase extracted from the liver of neon flying squid described here is a cysteine proteinase. This is in agreement with the nature of cysteine proteinases, because as a rule, these enzymes are highly sensitive to oxidizing and alkylating agents. They are also activated in the presence of chelating agents, e.g., EDTA, and reducing agents (Belitz and Grosch, 1987).

By taking these characteristics into account, we are now working on utilization of this enzyme through examining the reverse reaction, especially plastein reaction.

Acknowledgment

The authors wish to thank Mr. Michinori Takahashi for his help throughout this work.

References

Anonymous (1986). *Polyacrylamide Gel Electrophoresis : Laboratory techniques*. Pharmacia Fine Chemicals, Uppsala, Sweden.

Anson, M.L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.*, **22**, 79-89.

Belitz, H.D. and Grosch, W. (1987). *Food Chemistry*. Springer-Verlag, Heidelberg, Germany.

Exterkate, F.A. and De Veer, G.J.C.M. (1987). Complexity of the native cell wall proteinase of *Lactococcus lactis* subsp. *cremoris* HP and purification of the enzyme. *System Appl. Microbiol.*, **9**, 183-191.

Geis, A., Bockelmann, W. and Teuber, M. (1985). Simultaneous extraction and purification of a cell wall-associated peptidase and β-casein specific protease from *Streptococcus cremoris* AC1. *Appl. Microbiol. Biotechnol.*, **23**, 79-84.

Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, K. (1958). Crystalline bacterial proteinase. I. Preparation of crystalline proteinase of *Bacillus subtilis*. *J. Biochem.*, **45**, 185-194.

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen. Physiol.*, **30**, 291-310.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

Monnet, V., Le Bars, D. and Gripon, J.C. (1987). Purification and characterization of a cell wall proteinase from *Streptococcus lactis* NCDO 763. *J. Dairy Res.*, **54**, 247-255.