Studies on Muscle Alkaline Protease—I Isolation, Purification and Some Physicochemical Properties of an Alkaline Protease from Carp Muscle

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(Received August 30, 1973)

An alkaline protease was isolated from crude extract of carp ordinary muscle by ammonium sulfate fractionation (30–70% saturation), heat-treatment (58°C), DEAE-Sephadex A-50 column chromatography and Sepharose 6B gel filtration. The specific activity increased about 300 fold that of the crude extract with high recovery, and the purified preparation was homogeneous as indicated by ultracentrifugal ($S_{20,w}^0$: 19.3) and disc-electrophoretic analyses. The absorption spectrum of the purified enzyme was maximal at 278 m μ and minimal at 252 m μ . The specific extinction at 280 m μ ($E_{1\%}^{1\text{cm}}$) was 5.31. The molecular weight was found to be about 780,000 by Sepharose 6B gel filtration and the isoelectric point, 5.2 by isoelectric focusing.

This enzyme seems to be different from cathepsins when molecular weight and other physicochemical and enzymatic properties are compared.

When fish meat paste prepared from some kinds of fish muscle is heated at 60°–65°C, the elasticity of Kamaboko is lower than that heated at 80°–90°C. Such a lowering of the elasticity is termed “Modori”.

Mechanism of “Modori” phenomenon has been recently investigated. NIWA *et al.**** reported that such deterioration of elasticity might be due to the heat denaturation of protein rather than the hydrolysis by proteolytic enzymes in muscle. On the other hand, MAKINODAN *et al.* reported that the “Modori” phenomenon might be caused by a proteinase, which was found^{1,2)} in some fish muscle and that the enzyme of partially purified preparation^{3,4)} hydrolyzed casein at 60°–65°C in a slightly alkaline pH range, but did not hydrolyze it at physiological temperature.⁴⁾ However, many questions are remaining on the properties of the enzyme. Moreover, a clear explanation has not been entirely made on the role of the enzyme *in vivo*.

In our present study, the alkaline protease was purified from carp ordinary muscle according to method of MAKINODAN *et al.*⁴⁾ and IODICE *et al.*⁵⁾ in order to clarify its enzymatic characteristics.

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*** E. NIWA, G. NAKAJIMA, and M. MIYAKE: Paper No. 77–78 presented at the spring meeting of this society (1971).

Experimentals

Collection of specimens Carp, *Cyprinus carpio*, (3 kg and 35–39 cm) were obtained from a carp culturer. The fish was stunned on the head by a blow, bled, eviscerated, and immediately the ordinary muscle was used as starting materials.

Materials Rabbit γ -globulin was kindly provided by Dr. NAKASHIMA of our Faculty. Crystalline urease was prepared from Jack bean in our laboratory according to the method of UEHARA *et al.*⁶⁾ *N*-Carbobenzoxyl-L-glutamyl-L-phenylalanine (CBZ-Glu-Phe), *N*-carbobenzoxyl-L-glutamyl-L-tyrosine (CBZ-Glu-Tyr), benzoyl-L-arginine amide (BZ-Arg amide) and glycyl-L-phenylalanine amide (Gly-Phe amide) were purchased from the Protein Research Foundation, Osaka. Other chemical reagents were of analytical grade. Solutions were prepared with deionized and distilled water by all-glass apparatus. Buffer solutions were designated tentatively in this experiment as follows; the buffer A: 0.1 M KH_2PO_4 - 0.05 M $\text{Na}_2\text{B}_4\text{O}_7$ buffer solution, containing 1 mM EDTA, the buffer B: 0.01 M KH_2PO_4 - 0.005 M $\text{Na}_2\text{B}_4\text{O}_7$ buffer solution, containing 0.1 mM EDTA, the buffer C: 0.005 M KH_2PO_4 - 0.0025 M $\text{Na}_2\text{B}_4\text{O}_7$ buffer solution, containing 0.1 mM EDTA, the buffer D: 0.02 M KH_2PO_4 - 0.01 M $\text{Na}_2\text{B}_4\text{O}_7$ buffer solution, containing 0.2 mM EDTA.

Measurement of enzymatic activity The caseinolytic activity was measured by the modified method of HAGIHARA *et al.*⁷⁾ The substrate was prepared by dissolving Hammarsten casein in the buffer A, pH 8.3 at 65°C for 5 min. The assay mixture containing 3 ml of the buffer A, 1 ml of an enzyme solution and 1 ml of 5% casein solution was incubated at 65°C for 40 min. The reaction was stopped by adding 5 ml of TCA solution (0.11 M trichloroacetic acid-0.22 M sodium acetate-0.33 M acetic acid) and the mixture was kept at 37°C for 20 min. After filtering the mixture through Toyo No. 5c filter paper, the absorbance at 280 m μ of the filtrate was measured with a HITACHI Model 101 spectrophotometer. A control was run by incubating the enzyme in the buffer A without substrate, followed by the addition of the TCA solution and the substrate at the end of the incubation period. The absorbance at 280 m μ of the control was subtracted from that of the assay sample. The relationship between the increase of absorbance and the enzyme concentrations was established. The specific activity was expressed as the initial change in the absorbance at 280 m μ per min per mg of enzyme protein.

Cathepsin D activity was measured with the acid-denatured bovine hemoglobin as a substrate which was prepared by treating bovine hemoglobin with 0.06 N hydrochloric acid solution at 3°C for 2 hr and thereafter by dialysis overnight against cold distilled water. The assay mixture containing an enzyme solution, 3 ml of 0.1 M citric acid-0.2 M phosphate buffer, pH 3.0, and 1 ml of 5% substrate in a total volume of 5 ml, was incubated at

37°C for 60 min. The reaction was stopped by adding 2 ml of 10% TCA. The subsequent procedure of assay was the same as the routine procedure described above.

Cathepsin A activity was determined by the method described by IODICE *et al.*,⁵⁾ in which CBZ-Glu-Phe or CBZ-Glu-Tyr was used as a substrate. A reaction mixture consisted of an enzyme solution, 0.035 M substrate, 0.1 M citrate-0.1 M sodium hydroxide buffer, pH 5.0 and 0.01 M cysteine as an activator, was incubated for 24 hr at 37°C. The activity was estimated by measuring the amount of liberated phenylalanine or tyrosine according to the colorimetric ninhydrin procedure of ROSEN.⁸⁾

Cathepsins B and C activities were determined by the method of BOUMA *et al.*⁹⁾ with a minor modification. A reaction mixture consisted of 0.05 M BZ-Arg amide, 0.1 M citrate-0.1 M sodium hydroxide buffer, pH 6.3 and 0.1 M cysteine as an activator was incubated for 24 hr at 37°C. Cathepsin B activity was determined by measuring the liberated ammonia by the Conway microdiffusion method. Cathepsin C was measured in the similar way, using Gly-Phe amide and 0.01 M cysteine.

Determination of protein The protein concentration was determined either by the micro-biuret method^{10,11)} or by the method of LOWRY *et al.*,¹²⁾ using bovine serum albumin (fraction V) as the standard.

Electrophoresis Disc-electrophoresis was carried out with a 7.5 or 5.0% polyacrylamide gel column at pH 9.4, according to the method of NAGAI,¹³⁾ using an apparatus from MITSUMI Kagaku Sangyo Co. A 5.0% gel was photo-polymerized with riboflavin instead of ammonium persulfate.

Determination of the molecular weight The molecular weight of the purified enzyme was determined according to the method of ANDREWS.¹⁴⁾

Determination of isoelectric point The isoelectric focusing was carried out by the method of VESTERBERG *et al.*,¹⁵⁾ using a column (110 ml) devised at our laboratory based on a LKB column.

Ultracentrifugal analysis Ultracentrifugal studies were performed with a HITACHI Model UCA-IA or BECKMAN Spinco Model E analytical ultracentrifuge equipped with a shlieren optics. The sedimentation constant ($S_{20,w}^0$) was obtained by extrapolating the $S_{20,w}$ values at various concentrations of enzyme protein.

Purification procedure of alkaline protease All steps of the purification procedures were carried out at 3°–4°C unless otherwise indicated.

Preparation of muscle extract 450 g of ordinary muscle was minced and then homogenized with 900 ml of 2% potassium chloride solution by a homogenizer for 3 min. Immediately after centrifugation at $13,000 \times g$ for 45 min, the precipitate was extracted repeatedly with the same extractant (900 ml) overnight. After centrifugation the combined supernatants were dialyzed over a period of 24 hr against the buffer C, pH 6.5. Fraction I (1,620 ml) was obtained from the inner solution by centrifugation for 20 min

at $13,000\times g$ to remove the precipitate.

Ammonium sulfate fractionation Solid ammonium sulfate was added to Fraction I (1,620 ml) to make 30% saturation. After mechanical stirring for 2 hr, the precipitate was removed by centrifugation for 30 min at $13,000\times g$ and was discarded. Ammonium sulfate was slowly added to the supernatant to make 70% saturation. After stirring for 2 hr, the precipitate containing the enzyme was collected by centrifugation at $13,000\times g$. The precipitate was dissolved in the buffer B, pH 6.5, and dialyzed overnight against the same buffer to remove ammonium sulfate. The inner solution (Fraction II) was obtained.

Heat treatment Fraction II was neutralized to pH 7.0 with 1 N sodium hydroxide solution and was heated for 5 min after reaching 58°C in a 60°C water bath with stirring. After cooling, the suspension was centrifuged at $13,000\times g$ for 20 min to remove a large amount of the coagulated proteins and 425 ml of yellowish brown coloured supernatant (Fraction III) was obtained. In order to concentrate Fraction III solid ammonium sulfate was slowly added to make 70% saturation. After mechanical stirring for 2 hr and centrifugation at $13,000\times g$ for 30 min, the precipitate obtained was dissolved in the buffer C, pH 6.5. The solution was dialyzed overnight against the same buffer solution, and the supernatant (Fraction IV) was obtained by centrifugation of the inner solution to remove insoluble proteins appeared.

Results

The extraction conditions of an alkaline protease were preliminarily examined prior to the purification. The efficiency of extraction of the alkaline protease was investigated by measuring the enzymatic activity of extracts with distilled water, various concentrations (0.5 to 2.0%) of potassium chloride or the buffer C, pH 6.5 solutions, in comparison with that of cathepsin D. It was reported that cathepsin D is the most primary proteinase since it initially hydrolyzes a protein to peptide fragments which are further digested by other cathepsins such as A and C.^{5,16)} The alkaline protease was insufficiently extracted with distilled water, but enough with potassium chloride or the buffer C solution, while cathepsin D was extracted similarly with all the extractants tested. Therefore, 2% potassium chloride solution was used as an extractant in the following experiments.

The efficiency of repeating of extraction on solubilization of the alkaline protease and cathepsin D by 2% potassium chloride solution is shown in Table 1. The major part of the alkaline protease was recovered by twice extractions. The addition of 0.2% Triton X-100 in 2% potassium chloride solution was effective for the solubilization of cathepsin D, but not for the alkaline protease.

Effects of freezing-thawing of muscle homogenate was investigated on the extraction of the alkaline protease and cathepsin D. The repeat of this procedure was not effective

Table 1. Effect of repeat of extraction on solubilization of muscle alkaline protease and cathepsin D.

Minced muscle was extracted with 2 fold weight of 2% potassium chloride solution at 3°C for 1 hr and then immediately centrifuged at $13,000 \times g$ for 30 min. The fresh extractant was again added to the precipitate. The same extraction process was repeated 4 times (I to IV), and finally the precipitate was extracted by the same method with 2% potassium chloride solution containing 0.2% non-ionic detergent Triton X-100 (V*). Each supernatant obtained was dialyzed overnight against the buffer C, pH 6.5.

Enzyme	Proteolytic activity	I	II	Repeat of extraction III	IV	V*
Alkaline protease	Specific activity ($A_{280}/\text{min}/\text{mg}$)	4.23×10^{-3}	3.47×10^{-3}	1.61×10^{-3}	1.72×10^{-3}	0.91×10^{-3}
	Total activity (A_{280}/min)	78.9	18.2	2.80	1.61	3.17
Cathepsin D	Specific activity ($A_{280}/\text{min}/\text{mg}$)	1.25×10^{-3}	3.32×10^{-3}	3.42×10^{-3}	3.08×10^{-3}	6.03×10^{-3}
	Total activity (A_{280}/min)	23.3	17.4	5.96	2.88	20.9

for the extraction of the alkaline protease, but effective for that of cathepsin D.

On the effect of the heat treatment it was reported that the specific activity of the alkaline protease was increased, on the contrary the cathepsin D was inactivated.⁴⁾ In this report in order to investigate effects of the heat treatment at various temperature and pHs, the activity of the alkaline protease was determined and its result is shown in Fig. 1.

Fig. 1. Effect of heating at three different pHs on muscle alkaline protease.

Minced muscle was extracted with 2 fold weight of 2% potassium chloride solution and centrifuged at $13,000 \times g$ for 30 min. The supernatant was dialyzed overnight against the buffer C, pH 6.5 and then centrifuged to remove precipitated materials. The crude extract obtained was adjusted to pH 8.00, pH 6.78 or pH 4.00 with 1 N sodium hydroxide or hydrochloric acid solution. Each adjusted sample was divided into 4 parts and heated at various temperature for 5 min, and then centrifuged $13,000 \times g$ for 30 min to remove insoluble proteins denatured by heating. Alkaline protease activity and the amount of protein in the supernatants were determined.

×—× Protein, ○—○ Specific activity, ●—● Total activity.

When the extract was heated at high temperature (60°C) at neutral or slightly alkaline pH, the specific activities of the enzyme were increased, because a large amount of inactive proteins were coagulated and removed by centrifugation. The heat treatment was the most effective at neutral pH as shown in the center figure. At the acidic pH range the enzyme activity was remarkably lost as shown in the right figure.

On the basis of these results described above, the alkaline protease was purified from carp ordinary muscle. The specific activity and recovery of the enzyme activity at each step in purification procedures are summarized in Table 2.

Table 2. Purification of alkaline protease from carp muscle.

	Fraction	Volume (ml)	Total Protein (mg)	Specific Activity ($A_{280}/\text{min}/\text{mg}$)	Purification	Recovery (%)
I	Crude extract	1,620	25,758	3.93×10^{-3}	1.00	100
II	30–70% AS fraction	485	15,763	5.77×10^{-3}	1.47	90.1
III	Heated fraction	425	5,440	1.41×10^{-2}	3.59	75.9
IV	0–70% AS fraction	121	4,308	1.70×10^{-2}	4.33	72.5
V	First DEAE-Sephadex A-50 fraction	70	147	4.07×10^{-1}	104	59.2
VI	Second DEAE-Sephadex A-50 fraction	21.2	82.5	5.68×10^{-1}	150	46.4
VII	First sepharose 6B fraction	30.0	38.4	9.38×10^{-1}	239	35.6
VIII	Second Sepharose 6B fraction	—	—	1.17	298	—

The recovery of the activity at ammonium sulfate fractionation (30 to 70% saturation) of the crude extract was 90%, and the specific activity increased 1.5 fold at this step. By heat treatment of Fraction II at 58°C, pH 7.0, the specific activity increased 2.4 times and about 16% of the activity was lost. Cathepsin D was completely inactivated by this heat treatment as reported by MAKINODAN *et al.*,⁴⁾ because its activity found in the crude extract was not detected in this fraction.

An elution pattern of Fraction IV on a DEAE-Sephadex A-50 column is shown in Fig. 2. The alkaline protease activity was found only in the third peak (P-3). The specific activity of Fraction V obtained from P-3 increased 24 times and 82% of the activity was recovered at this step. Cathepsin A activity was found only in the second peak (P-2), and was 9% of the activity in the crude extract. The specific activity of cathepsin A in P-2 was 15 fold higher than that of the crude extract. Neither of cathepsins B, C and D activities was detected in all peaks.

The elution pattern of the second chromatography of Fraction V using a DEAE-Sephadex A-50 column is shown in Fig. 3. The activity of the alkaline protease was found in tube No. 35–54 (Fraction VI), whose specific activity was 150 fold higher than

Fig. 2. Chromatography of alkaline protease (Fraction IV) on the first DEAE-Sephadex A-50 column.

4,300 mg protein of Fraction IV (120 ml) was applied to a DEAE-Sephadex A-50 column (2.5×35 cm) which was previously equilibrated with the buffer B, pH 6.5. The same buffer solution was passed through the column until a large amount of yellowish inactive protein was completely eluted (P-1). And then the buffer B containing sodium chloride was stepwisely run. Contents in tube No. 88-132 were pooled (P-3). Fraction V was obtained by the ammonium sulfate precipitation of P-3 fraction and dialysis by the same method as described in the Fraction IV.

Eluent I: the buffer B, pH 6.5, II: the buffer B, pH 6.5, containing 0.2 M NaCl, III: the buffer B, pH 6.5, containing 0.4 M NaCl, IV: the buffer B, pH 6.5, containing 0.7 M NaCl, V: the buffer B, pH 8.6, containing 1.2 M NaCl. Flow rate: 5.1 ml/hr/cm².

Fig. 3. The second chromatography of the alkaline protease (Fraction V) on a DEAE-Sephadex A-50 column.

147 mg protein of Fraction V (70 ml) was applied to a DEAE-Sephadex A-50 column (1.5×26 cm) by the same way as the first chromatography. After washing the column with 30 ml of the buffer B, pH 6.5, the enzyme was eluted by a linear gradient of salt concentration from 0 to 0.6 M sodium chloride in the buffer B, pH 6.5. Contents in tube No. 35 to 54 were collected and Fraction VI was obtained by the ammonium sulfate precipitation and dialyzed in the same way as described in the Fraction IV.

Flow rate: 9.2 ml/hr/cm², — Absorbance at 280 mμ, ○—○ Proteolytic activity, ----- NaCl concentration.

that of the crude extract. A large part of inactive protein was removed at this step.

The result of the first gel filtration of Fraction VI on a Sepharose 6B column is shown in Fig. 4. The specific activities of contents in tube No. 16–21 (Fraction VII) were almost constant and were about 240 fold higher than that of the crude extract. And at this step the specific activity increased 1.7 times and 77% of the enzyme activity was recovered.

Fig. 4. The first gel filtration of the alkaline protease (Fraction VI) on a Sepharose 6B column. Fraction VI was subjected to a Sepharose 6B gel column (2.5×39 cm) which was previously equilibrated with the buffer D, pH 6.5. Elution was performed with the same buffer solution. Contents (Fraction VII) in 6 tubes (No. 16 to 21) were pooled. Flow rate: 1.9 ml/hr/cm², x—x Absorbance at 280 m μ , O—O Proteolytic activity, ●—● Specific activity, V_o : Void volume.

The result of the second gel filtration of Fraction VII is shown in Fig. 5. Both elution pattern of the enzyme activity and protein concentration were symmetrical, and the specific activity of samples in tube No. 14–16 (Fraction VIII) was absolutely constant and was about 300 fold higher than that of the crude extract. This fraction is homogeneous on an electrophoretic criterion, because only one band was observed on the disc-electrophoresis, shown in Fig. 6.

Fraction VIII was used as the purified alkaline protease on the study of the enzyme properties.

The result of the isoelectric focusing of Fraction VIII is shown in Fig. 7. A single peak of the enzyme activity and protein concentration was observed in the same fraction. The isoelectric point of the enzyme was revealed to be 5.2.

The ultraviolet absorption spectrum of the purified alkaline protease was determined in the buffer B, pH 6.5. The enzyme gave a typical protein ultraviolet absorption spectrum with the maximum absorption at 278 m μ and the minimum at 252 m μ , and the absorption ratio A_{278}/A_{252} was found to be 2.70. The specific extinction at 280 m μ ($E_{1\%}^{1\text{cm}}$) was 5.31.

Fig. 5. The second gel filtration of alkaline protease (Fraction VII) on a Sepharose 6B column. Into Fraction VII ammonium sulfate was added to make 70% saturation and was stored in a cold room (0°C). After centrifuging an aliquot of the stored enzyme preparation and dissolving the precipitate in a minimal amount of the buffer D, pH 6.5, about 5 ml of the enzyme solution was subjected to a longer 6B column (1.5×92.5 cm), as described in the first gel filtration procedure. Contents (Fraction VIII) in 3 tubes (No. 14 to 16) were pooled. Flow rate: 3.2 ml/hr/cm². Symbols are the same as in Fig. 4.

Fig. 6. Disc-electrophoresis of the purified alkaline protease.

The enzyme protein (20 μg) was placed on a 5.0% polyacrylamide gel column (0.5×7.0 cm) at pH 9.4 and permitted to migrate in an electric field of 2.0 ma current per tube for 2.5 hr. The gel was stained with coomassie blue.

Fig. 7. Isoelectric focusing pattern of the purified alkaline protease.

The protein (2.22 mg) was isoelectrically focused on the column containing carrier ampholyte (LKB producter AB) with a pH range 5 to 7 and sucrose. Electrophoresis was performed by the potential gradient of 700 volts for 44 hr, maintaining the column temperature at 2°C by circulating chilled water. After electrophoresis, the content in the column was fractionated into 2 ml aliquots. The pH of each fraction was measured with a HITACHI-HORIBA Model F-5 pH meter. The amount of protein and the caseinolytic activity in each fraction were measured.

--- pH of the fraction, ×× Absorbance at 280 mμ, ○—○ Caseinolytic activity.

Fig. 8. Determination of molecular weight of the purified alkaline protease by a Sepharose 6B gel filtration

The column (1.5×92.5 cm) was eluted with the buffer D, pH 6.5. The void volume was determined with blue dextran 2,000. Rabbit γ-globulin, Jack bean urease and porcine thyroglobulin were used as the standard proteins having the known molecular weight.

Molecular weight of the enzyme was determined by a Sepharose 6B gel filtration using γ -globulin, urease and thyroglobulin as controls as shown in Fig. 8. From the point of the elution volume of this enzyme on the standard curve, its molecular weight was determined to be 780,000.

Fig. 9. Ultracentrifuge patterns of the purified alkaline protease.

The solution contained 2.74 mg of protein/ml in the buffer B, pH 6.5, containing 0.1 M NaCl. Photographs, from right to left were taken at 6, 18, and 30 min after reaching full speed at 51,200 rpm, temperature 20 °C, phase angle 70°. The sedimentation coefficient, $S_{20,w}$ was calculated and found to be 18.9S.

Fig. 10. The dependence of sedimentation coefficients on the concentration of the enzyme protein. The sedimentation coefficient was determined on the four different concentrations of the enzyme protein, 2.56, 2.05, 1.24, and 0.65 mg/ml in the buffer B, pH 6.5 containing 0.1 M NaCl. The other conditions are the same as in Fig. 9.

$S_{20,w}$ values at infinite dilution.

By sedimentation analyses, Fraction VIII appeared to be homogeneous, showing a single symmetrical boundary in sedimentation velocity run at pH 6.5, with $S_{20,w}$ value of 18.9 by assuming the partial specific volume, $\bar{V}$ 0.74 (Fig. 9). The dependence of the sedimentation coefficients on the concentration of the enzyme protein is given in Fig. 10. $S_{20,w}$ values were almost constant at different concentrations of the enzyme protein. The sedimentation constant ($S_{20,w}^0$) of the enzyme was found to be 19.3 by extrapolating

Discussion

MAKINODAN *et al.*⁴⁾ reported that for the purification of alkaline protease the heat treatment at 63°–65°C at pH 8.0 at an early step was especially effective to remove inactive proteins, and that heating at lower temperature than 60°C was insufficient. However in this study, the heat treatment at 58°C at neutral pH was more effective on the

purification than that at slightly alkaline pH (8.0), as shown in Fig. 1.

It was reported that more than a half of the catheptic activities of rat skeletal muscle was found in myofibrillar protein fraction, but the extraction with a dilute saline solution containing 0.2% Triton X-100 could solubilize almost all of the catheptic activity bound with myofibrillar fraction.^{17,18)} As shown in Table 1, cathepsin D in carp muscle was slightly extracted by a dilute saline solution, but the alkaline protease was easily extracted. From these results the properties of carp muscular alkaline protease seem to be different from those of cathepsin D.

It was reported that cathepsins A, B, B', C, D, E, F and G exist in mammalian muscle,^{19,20)} kidney*, spleen,^{21,22,23,24)} liver,^{25,26)} thyroid²⁷⁾ or leucocyte.²⁸⁾ The molecular weight and the sedimentation constant of this enzyme were larger than those of these cathepsins reported.

Cathepsins A, B, C and D were also found in some kinds of fish muscle.^{29,30,31,32,33,34)} The difference between properties of this enzyme and those of cathepsins from fish muscle is to be discussed. Activity of cathepsins A, B, C and D was not detected in the purified alkaline protease preparation using their specific substrate. The optimal pH of this enzyme was 8.0–8.2 which is different from those of cathepsins A, B, C and D (5.0,²⁹⁾ 6.4,²⁹⁾ 6.4²⁹⁾ and 2.4–2.5³⁰⁾ or 4.3–4.6,^{32,33)} respectively). And the activity of this enzyme for 40 min incubation was the highest at 65°C which is also different from that of cathepsin D (at 50°C⁴⁾ or 42°C³⁰⁾). The molecular weight of this enzyme was 780,000 which is larger than that of cathepsin D from winter flounder and cod (32,000, 55,000, 65,000), reported by REDDI *et al.*³⁴⁾ and MUSCH *et al.*³³⁾ However, the molecular weight of this enzyme can not be compared with those of other cathepsins in fish muscle, because homogeneous preparation of them have not been reported.

Other properties of this enzyme will be presented in the succeeding paper.

On the dissociation of cathepsins, DOI *et al.** reported that two forms of cathepsin A (molecular weight 300,000 and 80,000) were isolated from porcine kidney. TURK *et al.*³⁵⁾ reported that when the purified cathepsin E preparation from beef spleen (molecular weight 305,000) was frozen and thawed and then treated with a Sephadex G-200 column, the activity of cathepsin D appeared in a smaller molecular fraction than that of cathepsin E, and this result suggested a possibility of change of cathepsin E to D *in vivo*.

From these reports, it is necessary to discuss a possibility of association or dissociation of the muscular alkaline protease *in vitro* or *in vivo*. Through the purification procedure in this study, especially by heat treatment, it may be considered that an unknown enzyme in the crude extract associates to this enzyme, because its molecular weight was

* E. DOI and C. OZURU: Paper No. 255 presented at annual meeting of the Agricultural Chemical Society of Japan (1972).

Fig. 11. Gel filtration of Fraction II on a Sepharose 6B column.

The sample was concentrated with ultrafiltration and 373 mg of protein (total enzymatic activity, 1.23) was loaded on a column (1.5×92.5 cm). The other conditions are the same as described in Fig. 5.

×—× Protein, ○—○ Proteolytic activity at 65°C, ●—● Proteolytic activity at 37°C.

considerably large.* In order to clarify this point, the gel filtration of Fraction II on a Sepharose 6B column, before heat treatment, was performed and the result is shown in Fig. 11. The alkaline protease was eluted at the same place where the purified enzyme was done, as shown in Fig. 5. From this result it is confirmed that molecular weight of the enzyme was not changed throughout the purification procedure.

Acknowledgements

The authors wish to thank Mr. K. KANNA of Tokai Regional Fisheries Research Laboratory and Miss H. KAWAKAMI of Cancer Research Laboratory, University of Kanazawa for the ultracentrifugal analyses and also Miss E. TOTSUKA for her excellent technical assistance. Thanks are also due to Dr. N. NAKAI and Mr. K. MITSUI for their helpful suggestions.

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