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THE QUATERNARY STRUCTURE OF CARP MUSCLE ALKALINE PROTEASE

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Summary

Carp muscle alkaline protease consists of four kinds of subunits, and its composition was assumed to be $(\alpha\beta\gamma_2\delta_2)_4$. It dissociated in the presence of 2-mercaptoethanol into an enzyme and α-subunits which upon removal of 2-mercaptoethanol rapidly aggregated to form a precipitate. The composition of the 2-mercaptoethanol-treated enzyme was $(\beta\gamma_2\delta_2)_4$. The pH of a 2-mercaptoethanol-treated enzyme solution was lowered to 4.5 by the addition of acetic acid in the presence of 0.4 M LiCl and centrifuged to separate the precipitate formed; this exhibited little activity and was mainly composed of β-subunits. The supernatant fluid recovered 53% of activity and contained an enzyme, whose composition was $(\gamma_4\delta_4)_4$. The temperature-activity curve of the native enzyme was the same as that of the 2-mercaptoethanol-treated enzyme and both were unable to hydrolyze casein at all below 55°C. However, the temperature dependence for activity of the LiCl-treated enzyme was ordinary: it hydrolyzed casein at physiological temperatures. When the 2-mercaptoethanol-treated enzyme was incubated with 4.5 M urea at 45°C for 20 min and this was followed by column chromatography, a little activity was recovered and the amount of recovery was parallel with the amount of δ-subunit in the fractions. These findings suggest; (1) the α-subunit does not take any part in activity but is a protein necessary for binding between subunits or between the enzyme and some functional proteins in the cells, (2) the β-subunit is used as

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Abbreviations: SDS, sodium dodecyl sulfate; EGTA, bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

inhibitor in the quaternary structure of the enzyme, (3) the δ-subunit is the catalytic one, and (4) binding with the γ-subunit is necessary for the δ-subunit to retain its active comformation.

Introduction

Makinodan et al. [1,2] have found proteases which hydrolyze casein in skeletal muscle of some fish. We [3—5] have reported the purification, and characterization of the carp enzyme and its distribution among fish. These were characterized by an optimum pH of 8.0, an optimum temperature of 65°C, no activity below 55°C and a high molecular size. We report here on the dissociation of carp muscle alkaline protease into four kinds of subunit and the role of each subunit in the activity.

Materials and Methods

Purification of the enzyme. This was done as reported earlier [3]. Fresh skeletal muscle of carp, *Cyprinus carpio*, was minced and extracted with 2% KCl. The enzyme was purified from the extract by ammonium sulfate precipitation (30—70% saturation), removal of proteins coagulated by heating at 58°C for 5 min, repeated column chromatography with a DEAE-Sephadex A-50 and repeated gel filtration on a Sepharose 6B column. During these procedures, the specific activity increased 300-fold, and the purified enzyme preparations were homogeneous, judged on the criteria of sedimentation, gel filtration and polyacrylamide gel electrophoresis.

Assay of enzymatic activity. The caseinolytic activity was measured by the method of Hagihara et al. [6] with a minor modification. Hammarsten casein was dissolved in 0.1 M phosphate/0.05 M borate buffer containing 1 mM EDTA (pH 8.3) by heating at 65°C for 5 min. An enzyme solution was added to 1 ml 5% casein and the total volume was adjusted to 3 ml by addition of buffer and incubated at 65°C for 40 min. After the addition of 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid, the reaction mixture was kept at 37°C for 40 min. After removal of a precipitate by filtration, absorbance at 280 nm of the filtrate was measured with a Hitachi Model 101 spectrophotometer. A control was run by incubating the enzyme solution without substrate, followed by addition of the trichloroacetate/acetate solution and the substrate. The absorbance of the control was subtracted from that of the sample. Specific activity of the purified enzyme preparations was 1.17 A_{280}/min per mg protein.

Determination of molecular weight. The sedimentation equilibrium method was performed, using a multichannel cell, in a Hitachi Model UCA-IA analytical ultracentrifuge equipped with schlieren optics [7]. The molecular weight of the enzyme was calculated to be 568 000 ± 8000, assuming the partial specific volume to be 0.75. It was estimated to be 600 000 by Sepharose 6B column chromatography, using myoglobin (17 200), hemoglobin (64 500), yeast alcohol dehydrogenase (150 000) and thyroglobin (660 000) as standard proteins.

Other methods. Gel electrophoresis in 7.5 or 5% polyacrylamide gels was run at pH 8.5 for 1.5 h at 3 mA/tube [8]. After this, the gels were stained with Coomassie brilliant blue. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed in 0.1% SDS, 10% polyacrylamide gels at pH 7.2 [9]. After electrophoresis and staining, absorbance at 600 nm of the gels was measured by density-scanning with a Gilford spectrophotometer Model 250. A linear relationship between intensity and amount of protein was observed under certain fixed conditions [10].

Isoelectric focusing was carried out at 4°C with Ampholines (pH 4—6 or 3.5—10) in a 0—30% (w/w) sucrose gradient, and was continued for 72 h with a maximum potential of 550 V [11].

Protein concentrations were measured using bovine serum albumin (Fraction V) as standard [12].

Materials

Bovine serum albumin (Fraction V), ovalbumin, yeast alcohol dehydrogenase and myoglobin were purchased from Sigma Chemical Co. (St. Louis). Horse heart cytochrome *c* was kindly provided by Sankyo Co. (Tokyo). DEAE-Sephadex A-50 and Sepharose 6B were purchased from Pharmacia Fine Chemicals (Uppsala). Hydroxyapatite was the product of Seikagaku-kogyo (Tokyo). All other chemicals were of the reagent grade and obtained from commercial sources.

Results

Three different preparations of purified carp muscle alkaline protease were shown to have a molecular weight of 600 000 by Sepharose 6B gel filtration, and another to have one of 568 000 by the sedimentation equilibrium method. The enzyme gave four bands on SDS-polyacrylamide gels after electrophoresis (Fig. 1), and the molecular weights of the subunits were estimated to be 36 000 (α), 32 000 (β), 24 000 (γ) and 18 000 (δ). Assuming that these subunits are similarly stained by Coomassie brilliant blue, an amount ratio between the sub-

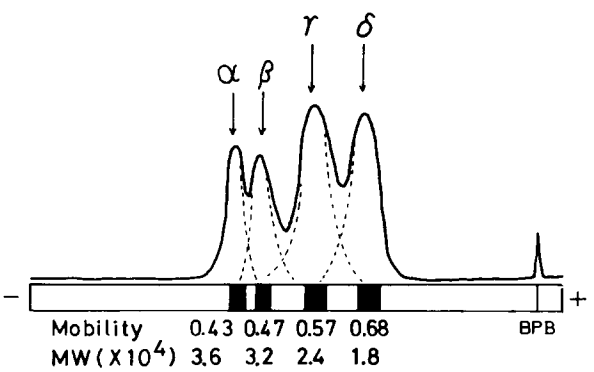


Fig. 1. SDS-polyacrylamide gel electrophoresis of alkaline protease.

TABLE I

COMPOSITION OF THE NATIVE ENZYME

The amount ratio between subunits was calculated from the density pattern of a SDS-polyacrylamide gel of the enzyme after electrophoresis, and the molar ratio was calculated from the molecular weight of each subunit and the amount ratio between subunits. Molecular weight of the enzyme was determined by gel filtration and calculated from the molar ratio between subunits and molecular weight of each subunit. The molecular weights of $\alpha_2(\beta\gamma_2\delta_2)_4$, $(\alpha\beta\gamma_2\delta_2)_4$ and $\alpha_6(\beta\gamma_2\delta_2)_4$ were calculated to be 536 600, 608 000 and 680 000, respectively.

No.	Amount ratio				Molar ratio				Molecular weight	
	α	β	γ	δ	α	β	γ	δ	Calcd.	Found
1	0.8	0.6	1.2	1.0	3.3	2.8	7.4	8.0	530 000	600 000
2	1.0	1.0	1.5	1.0	4.0	4.2	9.0	8.0	638 000	600 000
3	0.5	0.8	1.0	1.0	2.1	3.4	6.2	8.0	477 000	ND *
4	1.6	0.7	1.2	1.0	6.3	3.2	7.1	8.0	644 000	ND *
average						3.4	7.4	8.0		

* Not determined.

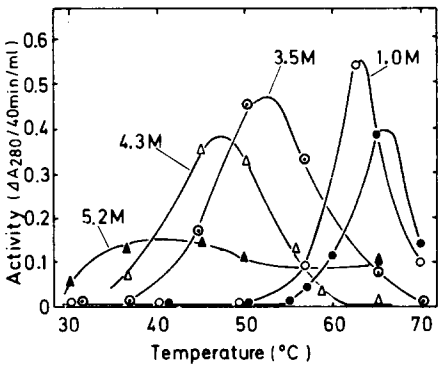
units was calculated from the density-scanning profiles of gel electrophoresis. The molar ratio between β-, γ- and δ-subunit, which was roughly constant in the four experiments, was 1 : 2 : 2 (Table I). However, the molar ratio of α-subunit to the other subunits varied from 0.5 to 1.5, suggesting some variation of molecular weight of the enzyme dependent on the amount of this subunit. Judging from the molecular weight of the enzyme and its subunits, composition of the enzyme was assumed to be $(\alpha\beta\gamma_2\delta_2)_4$, but it may occasionally be $\alpha_2(\beta\gamma_2\delta_2)_4$ or $\alpha_6(\beta\gamma_2\delta_2)_4$. The same enzyme purified in an earlier study [3] was shown to have a molecular weight of 680 000, suggesting that its composition is $\alpha_6(\beta\gamma_2\delta_2)_4$.

TABLE II

ENZYME ACTIVITY AT 65°C IN THE PRESENCE OF VARIOUS COMPOUNDS

No enzyme activity at 45 and 37°C was detected in the presence of the test compounds, except urea. In the presence of 4.5 M urea, enzyme activity was 92% at 45°C and 17% at 37°C of the enzyme activity at 65°C.

Compounds (concentration)	Relative activity
None	100
SDS (3 mM)	67
Tween-20 (1%)	100
Brij-35 (1%)	100
Ethanol (4%)	68
Urea (4.5 M)	23
Sodium trichloroacetate (4 M)	50
KCl (0.5 M)	24
LiCl (0.1 M)	19
LiBr (0.1 M)	27
Calcium acetate (1 mM)	68
NaSCN (0.1 M)	100
EGTA (1 mM)	117
2-Mercaptoethanol (1 mM)	123



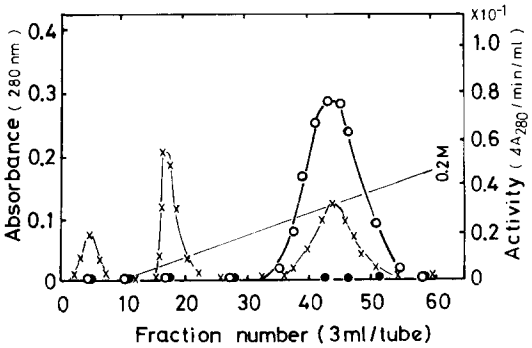


Fig. 2. Effect of urea on the enzyme activity. The enzyme (115 μg of protein) dissolved in 5 ml of 0.1 M phosphate/0.05 M borate buffer, pH 8.3, was assayed in the presence of urea in different concentrations and in the absence of urea under the conditions described in a previous report [3]. ●———●, in the absence of urea.

Fig. 3. Dissociation of the native enzyme by hydroxylapatite column chromatography in the presence of 2-mercaptoethanol. A hydroxylapatite column (1.1 × 4 cm) was equilibrated with 1 mM phosphate buffer (pH 6.9) containing 50 mM 2-mercaptoethanol. The enzyme (5.1 mg protein) was dissolved in 3 ml of the same buffer, applied to the column and eluted by the linear gradient of 1—200 mM phosphate. X- - - - -X, 280 nm; ○———○, activity at 65°C; ●———●, at 45°C.

The enzyme is unable to hydrolyze casein below 55°C. The effects of various compounds on this temperature dependence were examined (Table II). No activity could be detected at 37 or 45°C in the presence of all the test compounds except urea. Salts such as KCl, LiCl, LiBr and calcium acetate reversibly inhibited activity, and EDTA and 2-mercaptoethanol slightly activated the enzyme. Optimum temperature for activity was lowered by urea; at a high concentration, some activity appeared at 37°C, but this was relatively small (Fig. 2).

The enzyme was divided into three fractions by hydroxyapatite column chromatography in the presence of 2-mercaptoethanol (Fig. 3). The first fraction, eluted in the void volume, exhibited no activity and showed a single band on SDS-polyacrylamide gels after electrophoresis. Molecular weight of the protein in this fraction was determined to be 60 000 by gel filtration, but the fraction has not yet been identified. The second fraction, eluted at 30 mM phosphate buffer, exhibited no activity and contained α_4 and α_8, identified by gel filtration and SDS-polyacrylamide gel electrophoresis. During dialysis against 20 mM phosphate/10 mM borate buffer (pH 8.3) for 48 h, they aggregated into a precipitate. The last fraction, eluted at 120 mM phosphate, recovered 80% of activity and contained only an enzyme. The molecular weight of this enzyme was determined to be 480 000 by gel filtration, and the molar ratio between subunits was shown to be $\beta_{1.0}\gamma_{1.9}\delta_{2.0}$ by SDS-polyacrylamide gel electrophoresis (Fig. 4).

3 M LiCl was added to the 2-mercaptoethanol-treated enzyme dissolved in 2.6 ml 0.02 M phosphate/0.01 M borate buffer (pH 8.3), to a final concentration of 0.4 M and the pH of the solution was adjusted to 4.5 by adding 1 M acetic acid, with gentle stirring. After standing for 1 h at 4°C, the mixture was

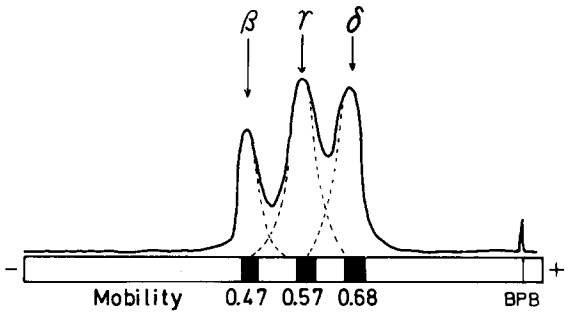


Fig. 4. SDS gel electrophoresis of the 2-mercaptoethanol-treated enzyme.

centrifuged to separate the precipitate thus formed. The precipitate contained only 4% of the activity and contained three kinds of subunit, of which the β-subunit was major, and the γ- and δ-subunit were minor components (Fig. 5). On the other hand, the supernatant fluid contained 53% of the activity and specific activity was increased 1.3-fold by this treatment. After the pH had been adjusted to 8.3 with 1 M NaOH, the supernatant fluid was immediately dialyzed against 0.02 M phosphate/0.01 M borate buffer (pH 8.3). The inner solution contained only an enzyme, consisting of equimolar amounts of γ- and δ-subunit (Fig. 5). Since its molecular weight was determined to be 650 000 by gel filtration, its composition was assumed to $(\gamma\delta)_{16}$. Its isoelectric point was determined to be pH 5.0 by isoelectric focusing and this is close to that of the native enzyme (5.2).

The native and the 2-mercaptoethanol-treated enzyme had similar catalytic properties. They were unable to hydrolyze casein below 55°C. However, the LiCl-treated enzyme exhibited activity even below 55°C, and its temperature-activity curve was normal, although the optimum temperature for activity was close to those of the others (Fig. 6), suggesting that the temperature stabilities are similar. Upon addition of the β-subunit, the LiCl-treated enzyme did not change its temperature dependence, suggesting that this dissociation is irrevers-

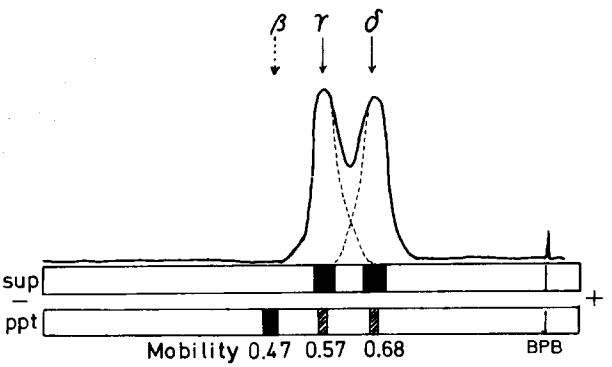


Fig. 5. SDS gel electrophoresis of the supernatant fluid (sup) and the precipitate (ppt) isolated from the 2-mercaptoethanol-treated enzyme by dissociation.

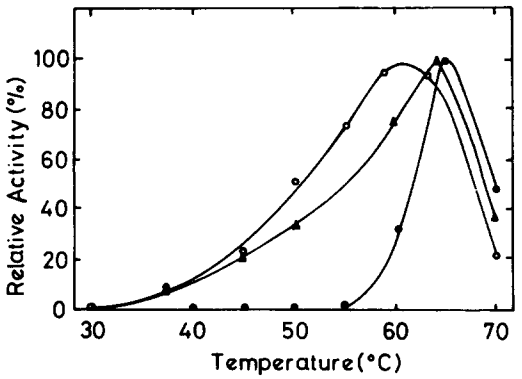
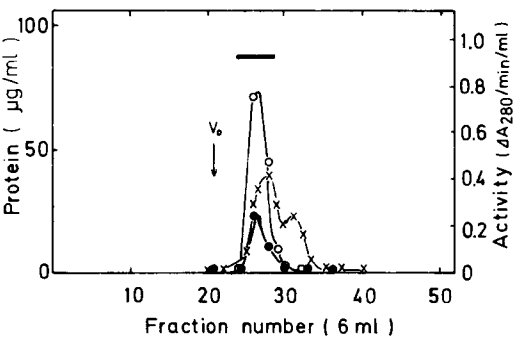


Fig. 6. Effect of reaction temperature on the activity of the native (●———●), 2-mercaptoethanol-treated enzyme (●———●), LiCl-treated enzyme (▲———▲) and the contents in tubes 25—29 in Fig. 7 (○———○).

ible. The optimum pH for activity of the LiCl-treated enzyme was 8.3, the same as that of the native and the 2-mercaptoethanol-treated enzymes. The LiCl-treated enzyme was relatively stable in a frozen state for several months, but a considerable amount of the enzyme aggregated. A small amount of the unchanged protein showed a single boundary which ran in sedimentation velocity with $s_{20,w}$ value of 20.7 S, which seems to support the estimates of 650 000 as the molecular weight of LiCl-treated enzyme, and $(\gamma\delta)_{16}$ as its composition. All attempts to dissociate the LiCl-treated enzyme into subunits under different conditions resulted in failure, suggesting that the catalytic subunit is unstable when not bound to another subunit.



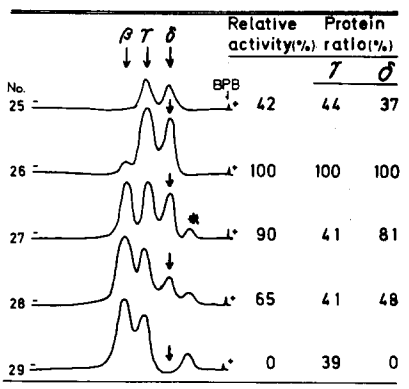


Fig. 7. Gel filtration of 2-mercaptoethanol-treated enzyme incubated with urea. Into the 2-mercaptoethanol-treated enzyme (7.2 mg) dissolved in 3 ml of 0.02 M phosphate/0.01 M borate buffer containing 1 mM EDTA (pH 8.3), was added 8 M urea to make a final concentration of 4.5 M and this was incubated at 45°C for 20 min. After incubation, the mixture was applied to a Sepharose 6B column (2.5 × 64 cm) equilibrated with the same buffer and eluted with the same buffer. X- - - - -X, protein; ○———○, activity at 65°C; ●———●, at 45°C.

Fig. 8. Relationship between the activity at 65°C and amounts of subunits of the content in tubes 25—29 in Fig. 7. Relative activity and amount of subunits in each tube are shown as percentages of the values of the content in tube 26. The star represents an unidentified protein.

After further treatment of the 2-mercaptoethanol-treated enzyme with 4.5 M urea at 45°C for 30 min, followed by gel filtration, 20% of the activity was recovered in fractions 25—29 (Fig. 7). The amount of activity was roughly parallel with the amount of δ-subunit in each tube, suggesting that this is the catalytic subunit (Fig. 8). The enzyme in this fraction was more active at lower temperatures than the LiCl-treated enzyme (Fig. 6).

Discussion

α-Subunit was released with ease from the native enzyme in the presence of 2-mercaptoethanol, and rapidly aggregated upon the removal of 2-mercaptoethanol. Its molar ratio to the other subunits varied among enzyme preparations and its release did not affect any catalytic property of the enzyme. These findings suggest that the α-subunit does not take any part in the activity, but is a protein necessary for binding between subunits in the enzyme molecule or between the enzyme and some functional proteins in muscle cells. The native and 2-mercaptoethanol-treated enzyme were unable to hydrolyze casein below 55°C, whereas the LiCl-treated enzyme showed an normal temperature-activity curve and was active at physiological temperatures, suggesting that the β-subunit is present as an inhibitor in the quarternary structure of the native and 2-mercaptoethanol-treated enzyme. However, the β-subunit did not inhibit the activity of the LiCl-treated enzyme. Therefore, a β-subunit-releasing mechanism would seem to be necessary for the activation of the enzyme in the cells. Results of experiments on urea treatment of the 2-mercaptoethanol-treated enzyme show that the δ-subunit is catalytic, and retains its active conformation when bound to the γ-subunit to form the LiCl-treated enzyme.

The native enzyme was shown to have tube-shaped structures as units of approx. 15 nm by negative staining electron microscopy; these consisted of four rough rings of about 10 nm external, and 2.5 nm internal, diameter. Most of the units were visible sporadically, and some bound together occasionally to form long tubes. Particles on the rings were too small to count however. Its composition of $(\alpha\beta\gamma_2\delta_2)_4$ and the four-layer structure suggest one ring consists of six particles such as $\alpha\beta\gamma_2\delta_2$. The LiCl-treated enzyme also revealed tube-shaped structures, similar to those of the native enzyme in size and shape, although the rings were seen to be smoother. This suggests that the rings consist of larger numbers of smaller particles and the structure of the enzyme is four octagonal layers, $(\gamma_4\delta_4)_4$. If this is so, one molecular of the LiCl-treated enzyme should be formed by reconstitution after dissociation of two molecules of the native enzyme, without the α- and β-subunit, via the 2-mercaptoethanol-treated enzyme during treatments.

Huston and Krebs [13] have found a phosphorylase b kinase-activating factor in rabbit muscle and suggest that it is a Ca^{2+}-activating protease. Busch et al. [14] have found a Ca^{2+}-activating protease in rabbit muscle and termed this Ca^{2+}-activated sarcoplasmic factor. It was purified from porcine [15,16] and chicken [17] skeletal muscle. Recently, Imahori [18] has reported that this enzyme was frequently inactivated by binding with a protein (molecular weight approx. 70 000) purified from another fraction in the enzyme purification procedure, and he suggested that the enzyme has some connection with

muscular dystrophy. Carp muscle alkaline protease was very slightly (1.12-fold) activated in the presence of 1 mM $CaCl_2$ [4]. The relationship between Ca^{2+}-activated muscle proteases of mammalia and muscle alkaline proteases of fish, and the β-subunit-releasing mechanism in the cells are interesting problems awaiting further elucidation.

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