CHEMICAL CHARACTERIZATION, ACTIVITY, AND THIOL CONTENT OF THE HIGHLY ACTIVE FORM OF CLOSTRIPAIN

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A highly active form of clostripain, composed of two polypeptide chains (M, = 43,000 and 12,500), was isolated by hydrophobic chromatography from the culture medium of Clostridium histolyticum. It differs in amino acid composition, namely in the value for cyst(e)ine, from that previously reported. The analyses of the separated chains are given.

Activity is related to the number of free cysteine residues and full activity is obtained only after complete reduction of the disulfide bonds. Specific modifications by sulfhydryl reagents and tosyl lysine chloromethyl ketone of one thiol group, the one implicated in the activity, are reported. High specificity of α-clostripain is restricted to arginyl peptide bonds as tested on parvalbumin.

Clostripain (EC 3.4.22.8) is a sulphydryl protease isolated from the culture filtrate of an anaerobic bacterium Clostridium histolyticum. Kocholaty et al. (1938) were the first to describe this enzyme as a cysteine-activated protease. This enzyme is specifically inhibited by sulphydryl reagents such as p-chloromercuribenzoate and, by analogy to papain, this thiol

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indicated of clostripain by the use of hydroxylapatite, first used by Gros and Labouesse, 1960). Ogle and Tytell (1953) noted its narrow specificity which is limited almost exclusively to arginyl esters or amides. Purification of clostripain by the use of hydroxylapatite, first used by Gros and Labouesse, 1960) and then by Mitchell and Harrington (1968), permitted the latter authors to study the specificity and the active site of clostripain with a more purified enzyme than has been described before. They introduced p-tosyl lysyl chloromethyl ketone as an inhibitor and suggested a possible role of a cysteine residue as a reacting site, in a similar way to papain (Porter et al., 1971). The catalytic site of clostripain may, therefore, have features in common with the catalytic sites of the papain, ficin, and bromelain family of sulphydryl enzymes, but the remarkable specificity of clostripain, limited to arginine residues, seemed to indicate an analogy of its binding site to that of trypsin.

Although in previous works, clostripain was described as chromatographically homogeneous (Gros and Labouesse, 1960; Mitchell and Harrington, 1968), Porter et al. (1971) reported that oxidative inactivation occurred during purification and that a substantial quantity of catalytically inactive enzyme was present in their best purified preparations of specific activity 125 units/mg. In 1973, Porter and Mitchell suggested the existence of two chromatographic conformers in apparently homogeneous, active clostripain due to the supposed formation of two separate and equally stable disulfide pairings. In 1977, Emod and Keil worked out purifications by affinity chromatography to obtain a highly active form of clostripain (specific activity, 270 units/mg) on a preparative scale. At the same time, pure p-nitrobenzoxycarbonyl-L-arginine chloromethyl ketone was synthesized and found to be a more specific inhibitor of clostripain than the corresponding L-lysyl chloromethyl ketone (Siffert et al., 1976; Keil, 1977). According to the studies of inhibition with this new titrant, a fraction of the highly purified enzyme seemed still to be in an inactive state.

In this study, the separation of clostripain into chromatographically homogeneous conformers, some without activity and one with a specific activity of 500 units/mg by means of chromatography on a ω-aminoalkylagarose column is described.

This highly active enzyme has a different amino acid composition and structural features from the preparations described previously and for these reasons, the name α-clostripain is proposed for this form of the enzyme. Affinity labeling and differential thiol group titrations gave some new insights into the structure-function relationships. The maximum specific activity is obtained when all disulfide bonds are reduced; however, only one —SH group, which can be selectively alkylated, is responsible for the catalytic activity.

EXPERIMENTAL PROCEDURES

The “Experimental Procedures” are presented in the miniprint supplement1 on p. 1467.

RESULTS

Exploratory Kits of Columns of Aminoalkylagarose—A series of ω-aminoalkylagarose columns (Sepharose-CnNH₂, n = 4 to 8) as described by Kula et al. (1976) was investigated for the purification of clostripain. Under our experimental conditions, the adsorption-elution profiles showed that clostripain was retained on all columns of this series and eluted by a gradient of sodium chloride. However, on a column of Sepharose-C₄NH₂, clostripain was eluted too rapidly, whereas on a column of Sepharose-C₅NH₂, the adsorption was too strong. The best resolutions were obtained on columns of Sepharose-C₆NH₂ or Sepharose-C₇NH₂ (Fig. 1). Contrary to the results of Kula et al. (1976), clostripain was not retained on homologous alkylagarose columns (Sepharose-Cₙ, where n =

1 Portions of this paper including “Experimental Procedures,” Figs. 1, 2, 3, and 7, and “Supplement References” are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-967, cite author(s), and include a check or money order for $1.50 per set of photocopies. The abbreviation used in the miniprint is: DTT, dithiothreitol.

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amidine gel under alkaline conditions (pH 8.5). It shows a single zone (Fig. 4). Furthermore, clostripain filtered through a Sephades G-10 column gave a single symmetrical peak of activity, apparently consistent with a molecular weight of 38,000 as estimated from the elution volume of standard proteins run on the same column (Fig. 5).

End group analysis gave two phenylthiohydantoins in an equimolar ratio: Ala and Asn. This was confirmed by the presence of two bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis on slabs as shown in Fig. 4. This pattern for the active clostripain was the same with or without β-mercaptoethanol. M, values of 43,000 and 12,500 were obtained by electrophoresis in polyacrylamide in SDS, as shown in Fig. 6. Thus, the apparent molecular weight of clostripain is approximately 55,000, a value in reasonable agreement with the value of approximately 50,000 determined by analytical ultracentrifugation of the native enzyme as described by Mitchell and Harrington (1968).

Separation of the Two Chains—In order to gain information concerning the two electrophoretic components, the reduced and S-pyridylethylated clostripain was chromatographed on a Sephadex G-75 column eluted with 9% formic acid. Two well separated components were obtained (Fig. 7), the heavy and light chains of α-clostripain.

The heavy chain corresponded to a molecular weight of 43,000 and it gave alanine as NH₂-terminal amino acid. The light chain had a molecular weight of approximately 12,500 and asparagine as NH₂-terminal amino acid.

Amino Acid Composition—The amino acid composition of pure α-clostripain is summarized in Table II. The sum of the amino acid compositions of the two isolated chains is in good accord with that of the whole protein. The analysis showed several differences from the amino acid content reported by Mitchell and Harrington (1968), but not from a commercial preparation of clostripain purified by us.

Specificity—According to several studies, clostripain cleaves proteins and synthetic substrates preferentially at the carboxyl group of arginine residues (Gros and Labouesse, 1960; Mitchell and Harrington, 1968). In view of several differences between our enzyme and that described previously, in amino acid composition, molecular weight, the presence of two chains, and activity, we have checked its specificity with the use of parvalbumin as substrate. Hake parvalbumin contains a single arginine residue and 12 lysine residues. Disulfide bonds are absent and the terminal amino group is acetylated. Thus, no traces of PTH-derivatives are formed when the protein is subjected to automatic Edman degradation. After an overnight digestion of parvalbumin by clostripain at 37°C, the resulting mixture was analyzed by sequential degradation. A new sequence (Ala-Leu-Thr-Asp) appeared as a consequence of the exclusive cleavage of the Arg-Ala bond. The results show clearly the strict specificity of clostripain for the arginyl bond; none of the 12 lysyl bonds were cleaved under our conditions.

Kinetic Constants and pH Optimum—The pH optimum of clostripain for the hydrolysis of α-N-benzyl arginine ethyl ester in phosphate buffer is about 7.0 to 7.2, which corresponds to the values obtained by Gros and Labouesse (1960) but is more sharply defined. The loss of activity under acidic conditions (pH 4.0) could be reversed by a change of pH towards the optimum pH.

The kinetic constants of our enzyme preparation were determined by a pH-stat titration on BAEE as substrate in phosphate buffer, pH 7.2. The apparent Michaelis constant 

\[ K_{m} \text{app} = 0.260 \text{mM} \]

is similar to that obtained by Porter et al. (1971) and Cole et al. (1971). The catalytic constant \( k_{cat} \) (580 s⁻¹) is more than 6-fold higher than that found by Ogle and
Fig. 5 (left). Estimation of the molecular weight of a-clostripain by gel filtration on a Sephadex G-100 column. The column (1.2 X 100 cm) was eluted with 50 mM Tris/HCl, 0.1 M NaCl pH 7.5 at 4°C. The column was calibrated with the protein standards: 1, myoglobin; 2, chymotrypsinogen; 3, ovalbumin; 4, bovine serum albumin. The samples were applied in 0.5 ml of buffer and 1-ml fractions were collected.

Fig. 6 (right). Mobility of the two chains of a-clostripain (1) and standard (0) on 10% acrylamide gels in SDS. The standard proteins were: 1, bovine serum albumin; 2, ovalbumin; 3, aldolase; 4, trypsinogen; 5, myoglobin; 6, cytochrome c; 7, parvalbumin.

TABLE II
Comparative amino acid analysis of various preparations of clostripain

| Amino acid       | a-Clostripain | Clostripain | Commercial clostripain | TLCK-modified clostripain | Heavy chain | Light chain |
|------------------|---------------|-------------|------------------------|---------------------------|-------------|-------------|
|                  | residues      |             |                        |                           |             |             |
| Histidine        | 9.1 ± 0.12    | 11.9        | 10.6                   | 9.2                       | 7.7         | 1.9         |
| Lysine           | 40.3 ± 0.12   | 44.6        | 48.4                   | 39.4                      | 31.3        | 10.7        |
| Arginine         | 11.2 ± 0.15   | 11.8        | 11.9                   | 11.5                      | 7.6         | 4.0         |
| Aspartic acid    | 82.4 ± 1.72   | 72.5        | 84.9                   | 86.4                      | 57.9        | 18.8        |
| Threonine        | 23.4 ± 0.06   | 24.0 ± 0.06 | 24.9 ±0.06             | 21.6 ±0.06                | 17.8 ±0.06  | 4.3 ±0.06   |
| Serine           | 36.7 ± 0.08   | 36.3 ± 0.08 | 36.8 ±0.08             | 33.8 ±0.08                | 28.3 ±0.08  | 7.2 ±0.08   |
| Glutamic acid    | 41.0 ± 0.11   | 48.0        | 48.6                   | 38.8                      | 10.7        |             |
| Proline          | 17.1 ± 0.68   | 14.7        | n.d.                   | 17.2                      | 15.6        | 4.9         |
| Glycine          | 40.4 ± 0.45   | 40.2        | 41.1                   | 39.3                      | 32.8        | 7.5         |
| Alanine          | 26 ± 0.04     | 25.0        | 26.0                   | 26.0                      | 20.0        | 6.0         |
| Valine           | 20.4 ± 0.21   | 23.0        | 23.4                   | 20.8                      | 17.2        | 4.5         |
| Methionine       | 10.4 ± 0.45   | 13.2        | 10.2                   | n.d.                      | 5.0         | 2.2         |
| Isoleucine       | 22.1 ± 0.57   | 21.0        | 26.3                   | 23.9                      | 17.5        | 6.5         |
| Leucine          | 37.7 ± 0.94   | 37.7        | 45.7                   | 40.8                      | 33.1        | 10.2        |
| Tyrosine         | 27.6 ± 0.29   | 17.1        | 27.8                   | 22.9 ±0.06                | 21.7        | 6.6         |
| Phenylalanine    | 18.2 ± 0.36   | 19.1        | 19.6                   | 19.5                      | 17.9        | 2.9         |
| Half-cystine     |               |             |                        |                           |             |             |
| CM-cysteine      | 6.6 ± 0.36    | n.d.        | 5.7                    | 0.97                      | 0           | 1-2         |
| PE-cysteine      | 10.5 ± 0.47   | 9.3         | n.d.                   | n.d.                      | n.d.        | n.d.        |

* Mean analyses (average of values) of 13 separate preparations, the residue ratios were calculated for $M_r = 55,000$.

° Clostripain from Mitchell and Harrington (1968).

°° corrected to zero time of hydrolysis.

°†, not corrected to zero time of hydrolysis.

° n.d., not determined.

* Arbitrarily taken as reference value for $M_r = 55,000$.

° CM-cysteine, carboxymethylated cysteine; PE-cysteine, S-pyridylethylated cysteine.

Tytell (1953), but it is similar to the theoretical $K_{cat}$ of 643 s$^{-1}$ deduced by Porter et al. (1971) on the basis of TLCK titration of the partly inactive enzyme. In our experiment, this value of $K_{cat}$ (580 s$^{-1}$), directly obtained, indicates a more homogenous, active enzyme than has been described before. However, in the absence of an appropriate direct titration of the active site, it may not be assumed that a higher value of $K_{cat}$ does not exist. This is a point which requires further investigation.

Thiol Number and Activation of Clostripain—As mentioned before, Porter and Mitchell (1973) assumed that the appearance of two distinct components of clostripain, after reduction by dithiothreitol and separation on an hydroxylapatite column, would indicate that the active site—SH groups were formed from at least two separate and equally stable
disulfide pairings, giving rise to two chromatographic conformers. As described above, we were able to separate the homogeneous preparation of clostripain obtained from a hydroxylapatite column on Sepharose-C-NH₂ into two forms of clostripain, one inactive and the other active, although both forms possessed the same NH₂-terminal amino acid residues. The activity was proportional to the extent of reductive activation.

The thiol contents of these two chromatographic components in various preparations of clostripain, activated to different extents, were determined in the presence and absence of 6 M guanidine HCl before and after reduction, as described by Weitzman (1976) (Table III). The same assay was performed with clostripain inactivated by oxidation. In the inactivated clostripain as in the enzyme from the Peak I of Sepharose-C-NH₂ chromatography, six thiol groups were involved in disulfide bonds and the free —SH group was buried inside the protein. In all active forms (Peak II), one thiol group was directly accessible, and the number of the thiol groups unmasked by denaturation was proportional to the activity. In the most active preparations, the direct titration after denaturation but without reduction showed that practically all the half-cystine residues present in clostripain are in the sulfhydryl form. This suggests that in the fully active form of clostripain, all the disulfide bonds are converted to free cysteine residues and that the disulfide bonds which could be titrated are experimental artifacts of the preparation.

Inhibition—The stringent sulfhydryl requirement for activity was studied by means of chemical modifications with various reagents. It should be noted, as a first point, that the inactivation due to the titration of the free —SH group of clostripain by DTNB under non-denaturing conditions is perfectly reversible; the addition of reducing reagent to DTNB-inhibited clostripain gave a 85 to 90% recovery of the initial activity. The result was similar to that obtained by Gros and Labouesse (1960) with the reversible inhibition of clostripain by p-chloromercuribenzoate. To distinguish the free —SH group which directly controls the activity from the other thiol groups formed by reduction in the active clostripain, different chemical modifications of cysteine residue were performed. In a first experiment after full activation of the native clostripain by dithiothreitol, accessible thiol groups were substituted by iodoacetic acid and this resulted in a complete loss of activity. The inactive protein was desalted on Sephadex G-25 and the other free cysteine residues liberated under denaturing and reducing conditions were modified by 4-vinyl pyridine. As can be seen in Table IV, 1 cysteine residue was transformed into S-carboxymethylcysteine, whereas the other thiol groups (5.8) were recovered as pyridylethylcysteine.

In a second experiment, the substitution of the fully active and native clostripain by TLCK resulted in a complete loss of activity. After being desalted, the inhibited protein was modified under denaturing and reducing conditions by iodoacetic acid. The results in Table IV show only 5.7 residues of CM-cysteine. Furthermore, no substitution of a histidine residue by TLCK was observed by amino acid analysis.

To determine whether TLCK and iodoacetic acid compete for the same site, a double modification was carried out; in one experiment, the native active protein was first reacted with TLCK and then radioactive iodoacetic acid was added. In a parallel experiment, the active clostripain was only substituted by [¹⁴C]iodoacetic acid. As the results in Table IV show, TLCK prevents the incorporation of radioactivity.

**DISCUSSION**

In all considerations of the relations between the structure and catalytic activity of clostripain, the problem of homogeneity plays an important role (Porter et al., 1971; Porter and Mitchell, 1973). Previous studies have shown that the exposure of the enzyme to reducing conditions brought about a considerable increase of activity. On the other hand, an extended reduction was never observed to bring about the loss of catalytic properties of the enzyme, due to the collapse of a preferential spatial arrangement stabilized by disulfide bridges. From the practical point of view of purification, the reversibility of sulfhydryl-disulfide conversion may produce a three-dimensional heterogeneity of a molecule which is sequentially homogeneous. Attempts to obtain the homogeneous enzyme were always undertaken by purification of its partially oxidized, and therefore partly inactive, forms. It was only by indirect evidence, based on the deactivation kinetics, that a much higher specific activity (300 units/mg) was deduced for the active enzyme than that obtained experimentally after its purification (Porter et al., 1971).

The chromatograph on α-aminoethylagarose of the reduced clostripain yields an enzyme of specific activity of at least 500 units/mg. This enzyme behaves as a homogeneous protein on gel chromatography and gives a single band on polyacrylamide gel electrophoresis. The SDS electrophoresis reveals the existence of three bands, two with an apparent molecular weight of 42,000 to 43,000, which, after reduction of the sample by mercaptoethanol, form a single band at M₅₀₀ = 43,000, and the lower band with an apparent molecular weight of 12,500. As we have never succeeded during our extensive purification efforts in separating active clostripain into two fractions corresponding to these two electrophoretic bands and as clostripain activity is always associated with the appearance of these two bands in equal molar ratios, we assume that the polypeptides in these 12,500- and 43,000-dalton bands...
are subunits of a parent 55- to 57,000-dalton clostripain molecule. The same two bands can be also found in commercial preparations of clostripain. The band of the light chain in SDS electrophoresis might escape detection as it is superposed on the band of the control dye.

The two chains of the fully reduced active clostripain are held together firmly by noncovalent bonds. Only after substitution of the cysteine residues under denaturing conditions, can they be separated by gel filtration. PTH-alanine and PTH-asparagine were found as NH- termini. In the previous contributions to this subject (Mitchell and Harrington, 1968), the presence of two subunits or chains in clostripain was not noted. The molecular weight of clostripain as a sum of the molecular weights of the subunits is in accordance with that obtained previously by ultracentrifugation (55,000) and gel filtration on Sephadex G-75 (50,000) (Mitchell and Harrington, 1968). On the contrary, gel filtration of either nonactivated, activated, or deactivated samples of clostripain on Sephadex G-100 always gave higher retention volumes which would indicate lower values for the apparent molecular weight, around 38,000. We have no explanation for this experimental result other than to assume a nonspecific adsorption to the gel.

The amino acid analysis of clostripain and those of the two isolated chains are the same. Our results for the intact enzyme differ from that obtained by Mitchell and Harrington (1968) with respect to two points. First, we find a 2-fold higher content of tyrosine residues which could explain the observed retardation of the protein on the Sephadex G-100 column and secondly, we find a lower number of cysteine residues. According to our analysis of oxidized, 4-pyridylethylated or carboxymethylated enzyme, clostripain contains 7 cysteine residues in contrast with 20 and 9 proposed in earlier studies (Mitchell and Harrington, 1968; Porter and Mitchell, 1973) for the molecular weight of 55,000. The amino acid analysis of a commercial sample of clostripain, purified by our methods, gave comparable values. Thus, the clostripain that we have isolated differs from that previously described. For these reasons, we propose the name a-clostripain for this well characterized form of clostripain.

The best known sulfhydryl protease, papain, is described as a protease with one sulfhydryl group at the active site and three disulfide bridges. However, Finkel and Smith (1958) suggested that fully active papain may not contain disulfide bridges and that, under certain conditions, as many as 6 mol of p-chloromercuribenzoate or p-chloromercuribenzene sulfonate react with papain. According to the present study, an analogous conclusion can be drawn for a-clostripain. Its activity is proportional to the number of free —SH groups: at least one accessible (see below) sulfhydryl group is indispensable for the appearance of activity and in the most active preparations that we could obtain, practically all the cysteine groups are free. We therefore assume that fully active clostripain has no disulfide bonds. On the other hand, one free sulfhydryl group exists both in the active and the inactive forms of clostripain, with the difference that in the inactive enzyme, it can be revealed only under denaturing conditions. There is presently no evidence that this free cysteine residue is the same in the two forms; one cannot exclude an interaction of the free —SH group with a disulfide bond during the activation process with a possible conformational change creating a catalytically competent active site. This assumption could explain the good separation of the active form of clostripain from the inactive form by ω-aminoalkylagarose chromatography.

Previous studies on activation by reducing agents and inhibition by thiol reagents accumulated strong evidence for the participation of a free —SH group in the activity of clostripain. The inhibition of the activity by modification of a thiol group with TLCK was first suggested by Porter et al. (1971). The same mechanism applies, presumably, for the specific inhibition of clostripain by p-nitrobenzoxycarbonyl-L-arginine chloromethyl ketone (Siffert et al., 1976). Our present study on reaction of a-clostripain with TLCK and iodoacetic acid supports this hypothesis: only 1 cysteine residue disappears after the reaction with TLCK and no histidine residue is modified; it follows that iodoacetic acid and TLCK compete either for the same thiol residue or for two neighboring thiol residues in the active form of a-clostripain. Only a sequence analysis of the modified cysteiny1 peptides will distinguish between these two possibilities.

The modification of a reactive cysteine residue in thiol protease by TLCK was demonstrated in papain by Whitaker and Perez-Villaseñor (1968) and in ficin by Marshall and Liener (1967). Yokosawa et al. (1977) succeeded in converting the hydroxyl group of the active serine of trypsin into a thiol group without a drastic change of the activity. In this thiol trypsin, TLCK substituted the new thiol group with subsequent loss of activity without modification of histidine residues; on the other hand, neither thiol trypsin nor clostripain or papain are inactivated by disopropyl fluorophosphate. A better understanding of the high specific affinity of clostripain for the arginine residues in proteins, its activation mechanism associated with cystine-cysteine rearrangements, and its eventual homology with other thiol proteinases must await more precise data on its primary and spatial structures.

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Supplemental References are found on p. 1467.
a-Clostrypain: chemical and enzymatic activity of the highly active form of clostrypain

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EXPERIMENTAL PROCEDURES

Materials:

Plasmodium berghei strain 7E1 cells were obtained from the Center for Disease Control, Atlanta, Georgia. The cells were maintained in culture in RPMI-1640 media supplemented with 10% fetal bovine serum. The parasites were harvested by centrifugation at 2000 rpm for 10 min and washed twice with 0.9% saline solution. The parasites were then resuspended in MEM containing 5% newborn calf serum and 0.01% penicillin G and streptomycin. The parasites were allowed to infect the mouse liver and were harvested for use in the experiments.

Enzyme activity:

The enzyme activity was determined by measuring the rate of substrate hydrolysis. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 10 mM calcium chloride, and 0.1 mM substrate. The mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 2 M HCl. The reaction products were then extracted with ethyl acetate and the absorbance of the organic phase was measured at 280 nm using a spectrophotometer.

Results:

The enzyme exhibited a broad specificity for various phospholipids, including phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. The enzyme activity was not affected by the presence of inhibitors, such as sodium fluoride, sodium pyrophosphate, and sodium arsenate.

Discussion:

The enzyme appears to be a novel enzyme that is involved in the degradation of phospholipids. The enzyme activity was not affected by the presence of inhibitors, which suggests that the enzyme is not a conventional phospholipase. The enzyme activity was not inhibited by a variety of conditions, which suggests that the enzyme is not a conventional phospholipase.

Conclusion:

The enzyme is a novel phospholipase that is involved in the degradation of phospholipids. The enzyme appears to be specific for various phospholipids and is not inhibited by the presence of inhibitors. Further studies are needed to determine the role of this enzyme in the degradation of phospholipids.
a-Clostripain

Fig. 1. Adsorption-elution profiles of crude clostripain on series of d-mannosyl-Sepharose columns of Sepharose G-75, 5 × 10 cm. After extraction and purification of the enzyme, it was applied to the column (60 ml) and eluted at a flow rate of 1 ml/min with a 0.1 M Tris-HCl pH 7.5 containing 0.1 M NaCl buffer. The buffer was then changed to Tris-HCl pH 7.5 containing 1 M NaCl (buffer B) and finally, with buffer C (buffer C and NaCl). Fractions of 10 ml were collected and monitored for activity (---) and absorbance at 280 nm (---). The fractions containing the enzyme were monitored.

Fig. 2. Hypo-osmotic column chromatography of a-1 aminopeptidase activity fraction. Protein was applied to the column (2.2 × 10 cm) in 0.1 M sodium phosphate buffer containing 0.2 M sucrose and eluted with a 0.1 M Tris-HCl pH 7.5 buffer containing 0.1 M NaCl. Fractions of 10 ml were collected and monitored for activity, and those which were pooled.

Fig. 3. Chromatography of heparin-Sepharose purified fraction on Sepharose G-75 column. After elution with a 0.1 M Tris-HCl buffer pH 7.5 containing 0.1 M NaCl, the column was eluted with a linear gradient (500 ml in each column) of salt (up to 2 M in the same buffer). The bars indicate the positions of activity. Peak 1 is an inactive form of clostripain. Peak 2 is the a-clostripain. The absorbance at 280 nm (---) and activity (---) were monitored.
alpha-Clostripain. Chemical characterization, activity, and thiol content of the highly active form of clostripain.

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