Chromatographic Evidence for the Existence of Multiple Forms of Cathepsin B1

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Preparations of ox spleen cathepsin B1 have been found to give multiple peaks of activity upon chromatography on DEAE-cellulose in NaCl gradients or by equilibrium chromatography in 0.05M-NaCl. CM-cellulose gradient chromatography also shows several cathepsin B1 peaks. This evidence indicates that ox spleen cathepsin B1 can exist in at least three to five forms. All forms have the same molecular weight, are thiol-activated and are inhibited by typical thiol inhibitors. The possible sources of this multiplicity of activity are discussed and a possible physiological role for cathepsin B2 is suggested.

The purification of cathepsin B was originally undertaken in this laboratory at a time when no chromatographic procedures were available. This activity had been partially purified by Greenbaum & Fruton (1957) by ethanol-mercury precipitation. While the present work was in progress, Otto (1967) reported the chromatographic separation of two enzymes with different molecular weights and with the ability to hydrolyse benzoyl-L-arginine amide. The smaller enzyme had a molecular weight of 24000 and was also able to hydrolyse benzoyl-DL-arginine *p*-nitroanilide. This enzyme has recently been called cathepsin B1 and the larger enzyme, with a molecular weight of 52000, has been called cathepsin B2 (Otto, 1971).

No definitive criteria of purity have been presented for any preparation of cathepsin B1. Otto (1967) and Otto & Bhakdi (1969) observed a symmetrical peak upon chromatography on Sephadex and one band on cellulose powder electrophoresis at pH4.65, a pH that is probably close to the isoelectric point of cathepsin B1. Snellman (1969) has reported on the purification of 'cathepsin B' and obtained a single species capable of hydrolysing benzoyl-L-arginine β -naphthylamide. Preparative polyacrylamide-gel electrophoresis, under conditions in which the protein emerged as a single peak but with no activity, and paper electrophoresis were the only indications of homogeneity. Keilova & Keil (1969) reported the separation of two proteins capable of hydrolysing benzoyl-L-arginine amide, but studied only one. These workers reported the presence of two *N*-terminal amino acids in their best preparations.

We have observed more than one form of cathepsin B1 upon gradient elution from DEAE-cellulose and CM-cellulose as well as upon equilibrium chromatography on DEAE-cellulose. The significance of these findings have led to this report on the chromatographic behaviour of cathepsin B1.

Experimental

Materials

Fresh ox spleens were obtained from a local slaughterhouse or a 40–70% satd.-(NH₄)₂SO₄ fraction prepared by the New England Enzyme Center (Boston, Mass., U.S.A.) was used. Special enzyme-grade ammonium sulphate (used in the laboratory-scale experiments), 2-mercaptoethanol, 2-mercaptoethylamine hydrochloride, benzoyl-L-arginine amide and benzoyl-DL-arginine *p*-nitroanilide were obtained from Mann Research Laboratories (New York, N.Y., U.S.A.). Dithiothreitol was purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and the thioglycollic acid was obtained from Evans Chemetics Inc. (New York, N.Y., U.S.A.). Iodoacetic acid, iodoacetamide and *N*-ethylmaleimide were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All buffers were prepared in distilled deionized water from reagent-grade chemicals.

Column chromatography

Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography was performed in 0.02M-sodium phosphate buffer, pH6.8, containing 0.02% sodium azide as preservative. Ion-exchange chromatography was performed on DEAE-cellulose (Whatman DE-52; Reeve Angel, Clifton, N.J., U.S.A.) in 0.02M-sodium phosphate buffer, pH6.8, or on CM-cellulose (Whatman CM-52) in 0.02M-sodium acetate buffer, pH5.0, containing 0.02% sodium azide. Active fractions are numbered in the order of elution by the gradient or salt solution, in equilibrium chromatography.

Assays for cathepsin B1 and B2

Hydrolysis of benzoyl-DL-arginine *p*-nitroanilide was followed by the method of Otto (1967) except

that a Coleman model 295 spectrophotometer with 10mm×75mm cells was used and 2-mercaptoethylamine was used in place of 2-mercaptoethanol. A unit of activity for the hydrolysis of benzoyl-DL-arginine *p*-nitroanilide is defined as the amount of enzyme which liberates 1 μ mol of *p*-nitroaniline/min. The assay of cathepsin B2 activity was performed by mixing 0.2 ml of 0.05 M-2-mercaptoethylamine hydrochloride, pH 5.0, and 0.5 ml of 0.1 M-benzoyl-L-arginine amide in 0.5 M-sodium citrate buffer, pH 5.0, with 0.3 ml of enzyme and incubating for 30–60 min at 37°C. At appropriate times a 0.1 ml sample was removed and tested for ammonia by the micro-distillation method of Seligson & Seligson (1951). A unit of cathepsin B2 activity is taken as that amount of enzyme which liberates 1 μ mol of ammonia/ml.

Determinations of molecular weight

The molecular weights of the enzymes were determined on a 1.5 cm×90 cm column of Sephadex G-100 as described by Whitaker (1963). The following proteins were used as standards: bovine serum albumin, ovalbumin, α -chymotrypsinogen A and cytochrome *c* (Sigma Chemical Co.), and Blue Dextran 2000 (Pharmacia Fine Chemicals) was used to determine the void volume.

Polyacrylamide-gel electrophoresis

Samples were run on 7% polyacrylamide slabs (Cyanogum 41; Fisher Scientific Co., Fair Lawn, N.J., U.S.A.) in an apparatus capable of holding slabs 12 cm×14 cm×0.5 cm. Both sides of the slab were cooled by circulating tap water. The pH was maintained by circulating the buffer between electrode compartments. Excess of ammonium persulphate catalyst was removed by pre-electrophoresis and by layering 0.7 μ mol of tris thioglycollate (Brewer, 1967) on each sample slot and allowing it to run ahead of the samples. Samples containing 100 μ g of protein were run for about 2 h at 100 mA (180 V). The gels were stained with 0.05% Amido Black (Buffalo Black NBR; Allied Chemical Co., Morristown, N.J., U.S.A.) in methanol – acetic acid – water (5:1:5, by vol.) for 20 min, destained electrophoretically in the same solvent and cleared in 50% glycerol.

N-Terminal analysis

The *N*-terminal residues of the cathepsin B1 fractions were determined by the fluorodinitrobenzene method (Fraenkel-Conrat *et al.*, 1955) on about 0.20 μ mol of each fraction. Standard Dnp-amino acids were commercial preparations (Mann Research Laboratories, New York, N.Y., U.S.A.) with the exception of Dnp-threonine, which was made by the modified aqueous procedure of Levy

(Fraenkel-Conrat *et al.*, 1955). Hydrolyses of the Dnp-enzyme derivatives were for 16 h in 6 M-HCl under vacuum. Thin-layer chromatography of the Dnp-amino acids was performed on activated silica gel-coated plastic plates (Eastman #6061, Rochester, N.Y., U.S.A.) and development was in an Eastman sandwich-type apparatus with solvent systems 3, 4 and 5 of Brenner *et al.* (1961). Pyridine was redistilled from KOH before use.

Determination of thiol groups

Thiol titrations were performed with 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma) as described by Ellman (1959). Typically, about 5 mg of enzyme in 0.5 ml of 0.1 M-sodium phosphate buffer, pH 6.5, was made 0.01 M in 2-mercaptoethanol and incubated for 15 min at 37°C. The activator was removed by passage through a 0.9 cm×20 cm column of Sephadex G-25 (Pharmacia Fine Chemicals) that was equilibrated with 0.10 M-sodium phosphate buffer, pH 8.0, which had been deaerated and flushed with N₂. The reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid) was complete after about 30 min as measured by the extinction at 412 nm at 23°C. An extinction coefficient of 13600 was assumed for the coloured product, 2-nitro-5-thiobenzoic acid (Ellman, 1959).

Concentration of protein

The protein concentration was determined by the method of Lowry *et al.* (1951) by using bovine serum albumin (Sigma Chemical Co.) as the standard, or by measuring the extinction at 280 nm, an extinction of 1.5 being assumed for a solution containing 1 mg of protein/ml (Otto, 1971).

Results and Discussion

The initial purification through the 40–70%-satd.-(NH₄)₂SO₄ fraction was performed essentially as described by Metrione *et al.* (1966) except that it was found that brief incubations, as short as 2 h, at room temperature were as efficient in terms of the total yield of cathepsins B and C recovered as the longer times and elevated temperature previously used.

The 40–70%-satd.-(NH₄)₂SO₄ fraction (60 ml) was then applied to a 5 cm×95 cm Sephadex G-100 column which had been equilibrated with 0.02 M-sodium phosphate buffer, pH 6.8 (Fig. 1). The cathepsin C activity was eluted near the void volume and was cleanly separated from cathepsins B1 and B2. The cathepsin C can then be further purified by the remaining steps of the earlier procedure (Metrione *et al.*, 1966).

Cathepsins B1 and B2 appeared as two peaks of activity under a single protein peak. Further

separation of these components can be obtained by applying smaller samples to the column, but this is not practical if large quantities of enzyme are being processed.

The pooled fractions containing the cathepsin B1 and B2 from four to six Sephadex columns were applied without concentration of the protein to a DEAE-cellulose column that had been equilibrated with 0.02M-sodium phosphate buffer, pH6.8. The column was eluted with the equilibrating buffer until the extinction at 280nm decreased sharply, indicating that the unbound material had passed through the column. Elution was accomplished with a linear gradient composed of 1000ml of 0.02M-sodium phosphate buffer, pH6.8, and 1000ml of the same buffer containing 0.6M-NaCl. A typical elution profile is shown in Fig. 2. Typically, 37% of the activity applied to the column was recovered in fraction 1, 27% in fraction 2 and 12% in fraction 3.

The cathepsin B activity was divided into three peaks. Two of these peaks also contain hydrolase activity towards benzoyl-DL-arginine *p*-nitroanilide, and on this basis can be considered to be similar to cathepsin B1 of Otto (1967).

These activities were further fractionated by the use of more shallow gradients and finally by equilibrium chromatography on DEAE-cellulose. In the latter experiments, as much as 750mg of the Sephadex fraction, which contained only the second peak of benzoyl-L-arginine amide- and benzoyl-DL-

arginine *p*-nitroanilide-hydrolysing activity from Sephadex G-100, i.e. the material of lower molecular weight, could be applied to a 0.9cm×20cm column equilibrated with 0.02M-sodium phosphate buffer, pH6.8. About half of the material applied remained as a dark band 2-3cm long at the top of the column, and the remaining protein washed through without binding to the column. After all of the unbound protein has been washed from the column, as monitored by measurement of extinction at 280nm, buffer containing 0.05M-NaCl was applied to the column. Under these conditions, at least three, and with some preparations four, peaks of benzoyl-DL-arginine *p*-nitroanilide-hydrolysing activity were eluted, as shown in Fig. 3. The fourth component eluted in 0.05M-NaCl is not found in all preparations. In addition, the wash peak eluted with dilute buffer is also capable of hydrolysing benzoyl-DL-arginine *p*-nitroanilide, thus accounting for four to five components with cathepsin B1 activity. The breakthrough peak was found to be heterogeneous and was not studied further. The four components eluted with 0.05M-NaCl have molecular weights of 27000, as determined on Sephadex G-100, in good agreement with Otto's (1967) value of 24000 for cathepsin B1. Thiol titrations with 5,5'-dithiobis-(2-nitrobenzoic acid) indicate the presence of 0.90mol

Fig. 1. Purification of cathepsins B on Sephadex G-100

The column was 5cm×95cm and the flow rate was approx. 90ml/h. The eluting buffer was 0.02M-sodium phosphate, pH6.8. Fractions of 20ml were collected. The 40-70%-satd.-(NH₄)₂SO₄ fraction (50ml) contained 50mg of protein/ml. The material eluting between 500ml and 720ml was pooled. —, *E*₂₈₀; ■, dipeptidyltransferase activity; ○, benzoyl-L-arginine amide hydrolysis; ●, benzoyl-DL-arginine *p*-nitroanilide hydrolysis.

Fig. 2. Separation of cathepsins B on DEAE-cellulose

The column was 5cm×35cm and the flow rate was approx. 90ml/h. The sample was applied as it was eluted from a series of Sephadex G-100 columns (Fig. 1); the volume was approx. 400ml. (a) Profile of extinction at 280nm (the straight line depicts the gradient). (b) Detail from the region of (a) that contained the enzymic activity. Fractions of 5ml were collected. Fractions were pooled as follows: fraction 1, tubes 106-112; fraction 2, tubes 113-124; fraction 3, tubes 130-138. —, *E*₂₈₀; ○, benzoyl-L-arginine amide hydrolysis; ●, benzoyl-DL-arginine *p*-nitroanilide hydrolysis.

Fig. 3. Separation of cathepsins B1 by DEAE-cellulose equilibrium chromatography

A portion (400mg) of Sephadex fraction was applied to a 0.9cm $\times$ 20cm column. The elution buffer was 0.02M-sodium phosphate, pH6.8, containing 0.05M-NaCl, beginning at the arrow. The flow rate was approx. 20ml/h; 5ml fractions were collected. Fractions were pooled as follows: fraction 1, between 115 and 130ml; fraction 2, between 140 and 175ml; fraction 3, between 175 and 185ml; fraction 4, between 185 and 215ml. —, E_{280} ; ●, benzoyl-DL-arginine *p*-nitroanilide-hydrolysing activity.

of thiol/mol of enzyme, also in accord with Otto (1971). Furthermore, fractions 3 and 4 appear to be homogeneous by gel filtration on Sephadex G-100 and >95% pure by polyacrylamide-gel electrophoresis at pH6.5 (Fig. 5) whereas fraction 2 contained about 30% of minor impurity under these conditions. Fraction 1 contained approx. 40% impurity by both methods. Each component had *N*-terminal leucine (or isoleucine) with no more than 5% contamination by other amino acids, as determined by the dinitrophenyl method. In a typical experiment, 13% of the activity was recovered in fraction 1, 30% in fraction 2 and 10% in fraction 3. A total of 87% of the activity applied to the column could be obtained by the use of an additional step of 0.12M-NaCl in 0.02M-sodium phosphate, pH6.8. The characterization of the material eluted with the higher salt concentration has not been undertaken. Typical specific activities of fractions were: 1, 1.86; 2, 4.24; 3, 5.70; 4, 4.62 units/mg.

The Sephadex fractions can also be further purified on a CM-cellulose column. As much as 550mg has been applied to a 0.9cm $\times$ 20cm CM-cellulose column that had been equilibrated with 0.02M-sodium acetate buffer, pH5.0. After the breakthrough peak was eluted, a linear gradient composed of 125ml each of dilute buffer and buffer containing 0.20M-NaCl was applied (Fig. 4). Three peaks of benzoyl-DL-arginine *p*-nitroanilide-hydrolysing activity were eluted in this gradient with some activity also in

Fig. 4. Separation of cathepsins B1 on CM-cellulose

A portion (520mg) of Sephadex fraction was applied to a 0.9cm $\times$ 20cm column. Elution was by the gradient described in the Results and Discussion section. The flow rate was approx. 20ml/h; 5ml fractions were collected. Fractions were pooled as follows: fraction 1, between 75 and 110ml; fraction 2, between 145 and 185ml; fraction 3, between 185 and 215ml. —, E_{280} ; ●, benzoyl-DL-arginine *p*-nitroanilide-hydrolysing activity. The sloping straight line depicts the gradient.

the breakthrough peak. The molecular weight of the active component of each fraction was 27000. The second fraction showed a single symmetrical peak in Sephadex G-100 gel filtration and approximately 10% impurity on polyacrylamide-gel electrophoresis at pH6.5. This method of fractionating the various components is capable of yielding material with 10% or less of contaminants (Fig. 5), although there is a great deal of variation in purity found, depending upon which crude preparation is used to start the purification. Although attempts at controlling the procedure to yield uniform final products have been only partly successful, each batch of crude enzyme yields a reproducible product through the chromatographic procedures. Typically, 7.6% of the activity applied to the column was recovered in fraction 1 and 78% in fraction 2. The CM-cellulose procedure has the advantage of relatively high recoveries of protein in the active fractions, and the use of DEAE-cellulose columns seems to result in a 'smearing' of activity between fractions with resultant losses. It may be worth noting that fraction 1 from DEAE-cellulose is eluted essentially in the position of fraction 2 on CM-cellulose, whereas DEAE-cellulose fraction 2 is eluted as CM-cellulose fraction 1. Typical specific activities by fractions were: 1, 4.02; 2, 13.2; 3, 0.165 units/mg.

Evidence has been presented which supports the view that at least three, and perhaps as many as five, components possessing the characteristics of cathepsin B1 (molecular weight 27000 and ability to hydrolyse benzoyl-DL-arginine *p*-nitroanilide) exist

and can be separated on DEAE-cellulose or CM-cellulose. The procedure reported by Otto (1967) and Snellman (1969) would not be expected to separate these components. In Otto's (1967) procedure, in which the ammonium sulphate fraction is applied to Amberlite IRC-50, all the cathepsins apparently

Fig. 5. Polyacrylamide-gel electrophoresis of DEAE-cellulose equilibrium fractions and CM-cellulose fractions

Fractions: 1, DEAE fraction 1; 2, DEAE fraction 2; 3, DEAE fraction 3; 4, DEAE fraction 4; 5, CM fraction 1; 6, CM fraction 2; 7, CM fraction 3. The electrophoresis was performed as described in the Experimental section after concentration of the fractions with an Amicon ultrafiltration apparatus with a UM-10 membrane.

wash through the column, emerging as a large protein peak near the void volume. This procedure is followed by chromatography of the active fractions on a Sephadex G-75 superfine column. Snellman (1969) used only Sephadex G-200 and G-100. Furthermore, Snellman (1969) used calf liver and a very different extraction procedure, with an acetone-dried powder, so that direct comparisons may not be possible.

Keilova & Keil (1969) used DEAE-Sephadex A-50 in addition to Sephadex G-100 in their purification procedure and did report the separation of two benzoyl-DL-arginine *p*-nitroanilide-hydrolysing components, one washing through the column in dilute buffer and the other requiring 0.1–0.2M-NaCl for elution. Only the latter component was studied, however. These workers apparently used stepwise elution, a procedure which is convenient for processing material rapidly but not ideal for maximum resolution of similar components. Therefore although this procedure was very useful for the preparation of material to study the specificity of cathepsin B on the β -chain of oxidized insulin (Keilova & Keil, 1969), it apparently was not capable of resolving a variety of components that may have been present in their preparations.

All the active fractions from the DEAE-cellulose columns reported in this paper required thiol activation. Typical results for the fractions from a DEAE-cellulose column eluted by gradient chromatography are shown in Table 1. Clearly, mercaptoethylamine and Cleland's reagent are somewhat more

Table 1. Effect of activators on the activities of the DEAE fractions shown in Fig. 2

The reaction mixtures were as described in the text except that other activators were substituted for the standard activators at the same concentrations, as indicated. The enzyme concentrations were 0.42mg/ml for the benzoyl-L-arginine amide (BAA) assays and 0.96mg/ml for the benzoyl-DL-arginine *p*-nitroanilide (BAPA) assays.

Fraction no.	Activator	Specific activity (units/mg)	
		BAA assay	BAPA assay
1	Mercaptoethylamine	5.95	2.49
	Mercaptoethanol	4.21	2.01
	Dithiothreitol	6.29	1.25
	Thioglycollic acid	5.05	1.19
2	Mercaptoethylamine	4.21	1.69
	Mercaptoethanol	2.50	1.15
	Dithiothreitol	3.50	1.39
	Thioglycollic acid	2.76	0.76
3	Mercaptoethylamine	8.07	0.68
	Mercaptoethanol	2.52	0.50
	Dithiothreitol	6.95	0.73
	Thioglycollic acid	3.45	0.43

Table 2. *Effect of inhibitors on the activities of the DEAE fractions shown in Fig. 2*

The enzyme (1.75mg) and inhibitor (1mM) were mixed in a final volume of 0.5ml and incubated for 30min at 37°C. Portions were then removed for assays [0.1ml for benzoyl-L-arginine amide (BAA) assay and 0.2ml for benzoyl-DL-arginine *p*-nitroanilide (BAPA) assays]. The enzyme concentrations were 0.35mg/ml for the BAA assays and 0.70mg/ml for the BAPA assays.

Fraction no.	Inhibitor	Specific activity (units/mg)	
		BAA assay	BAPA assay
1	None	6.82	2.59
	Iodoacetic acid	0.00	0.44
	Iodoacetamide	0.40	0.91
	<i>N</i> -Ethylmaleimide	2.94	1.39
2	None	5.83	2.53
	Iodoacetic acid	0.00	1.74
	Iodoacetamide	0.40	2.46
	<i>N</i> -Ethylmaleimide	2.77	2.46
3	None	6.03	—
	Iodoacetic acid	0.00	—
	Iodoacetamide	1.31	—
	<i>N</i> -Ethylmaleimide	3.43	—

effective than mercaptoethanol and thioglycollic acid. Similarly, all fractions are inhibited by thiol inhibitors, as shown in Table 2. Iodoacetic acid is more effective than iodoacetamide in inhibiting all three fractions. This is in agreement with the findings of Snellman (1971). *N*-Ethylmaleimide is less effective than either of the iodo compounds in all cases.

The results reported here suggest that cathepsin B1 may be composed of isoenzymes. It is possible that these isoenzymes represent genetic variants arising from the use of spleens from unknown and presumably mongrel cattle. It is also possible that a single form of the enzyme could be isolated from individual spleens or spleens from inbred strains of cattle. Such a situation has been previously observed for leucine aminopeptidase by Himmelhoch & Peterson (1968). However, the use of either of these sources is not practical for the preparation of large amounts of enzyme.

Another possibility is that these enzymically active components are artifacts resulting from the isolation procedure(s). However, it was found that working through the procedure very rapidly (with avoidance of lengthy dialysis, sample storage etc.) did not lower the number of forms isolated. Also investigated was the possibility that these components represented differential oxidation states of the cysteine residues of a single enzyme species. Incubation of the Sephadex fraction containing cathepsin B1 with Cleland's reagent (0.02M) for 4h at room temperature and subsequent equilibrium chromatography on DEAE-cellulose performed in

the presence of Cleland's reagent (1mM) did not change the appearance of the elution profile, as shown in Fig. 4. Elevating the temperature to 37°C and increasing the molarity of the Cleland's reagent to 0.1M also failed to affect the profile, although this treatment destroyed the activity. Enhancing the oxidation of the enzyme by adjusting the pH of the cathepsin B1-containing Sephadex fraction to 8.7 with 0.2M-tris did not cause any significant change in the absorption profile at 280nm upon equilibrium chromatography; however, this treatment also destroyed the activity. It therefore appears that multiple forms of cathepsin B1 do not result from reversible oxidation-reduction phenomena. The possibility of an artifactual origin for these components cannot be ruled out, however.

In any event, it appears likely that preparations of cathepsin B1 reported to be homogeneous by earlier workers actually contain mixtures of the various components described here. The nature and the extent of the actual structural differences among these enzymes is not known at this time.

Cathepsin B1 is an efficient protease whereas cathepsin B2, which is larger in size, has either limited or no protease activity (Otto, 1971). One can speculate therefore that cathepsin B2 is a zymogen of cathepsin B1 in which the active site is in a form similar to the active site of cathepsin B1, i.e. a 'preactive site', but is incapable of accepting the larger substrates such as proteins and bulky synthetic substrates (benzoyl-DL-arginine *p*-nitroanilide, benzoyl-L-arginine β -naphthylamide) owing to steric

factors, but can accept the smaller substrate, benzoyl-L-arginine amide. Otto (1971) has reported that there is no conversion of cathepsin B2 into cathepsin B1 by cysteine. Keilova & Keil (1969), however, have reported a conversion of a 40000-molecular-weight component into a 24000-molecular-weight component. We have confirmed Otto's findings that any activation of a zymogen cathepsin B2 does not occur through reduction of disulphide bonds. Activation of proteases usually occurs by cleavage of peptide bonds. A process analogous to that occurring during activation of the proteases of the digestive tract, i.e. peptide-bond cleavage, may also occur with the intracellular proteases. This could also account for the multiple forms of the enzyme. Chymotrypsinogen is activated to give three forms of chymotrypsin (Bettleheim & Neurath, 1955). If the activation of cathepsin B1 occurs by cleavage of peptide bonds near each other and near the C-terminal of the chains, no difference in molecular weight would be observed by the methods employed thus far, and the same N-terminal would be seen as reported here, and the small peptide liberated during activation of the zymogen might well be lost during the isolation of the enzyme.

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