PURIFICATION AND PROPERTIES OF PEPSTATIN HYDROLASE FROM *BACILLUS SPHAERICUS*

Sir:

Pepstatin A,[1,2] which is a specific inhibitor of acid proteases and has the structure: *iso*-valeryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid (*iso*-valeryl-Val-Val-AHMHA-Ala-AHMHA), has been found to be hydrolyzed by the toluenized cells or the cell-free extract from *Bacillus sphaericus* to *iso*-valeric acid, valine and Val-AHMHA-Ala-AHMHA as previously reported.[3] In this communication we report purification and characterization of this pepstatin-hydrolyzing enzyme which we tentatively named pepstatin hydrolase.

The enzyme activity was assayed by the liberation of *iso*-valeric acid from pepstatin A. A standard reaction mixture contained 0.3 μmoles of pepstatin A, 20 μmoles of phosphate buffer, pH 7.0 and an enzyme solution in a final volume of 0.2 ml. After 30 minutes at 37°C, the reaction was terminated by cooling in an ice bath. To the reaction mixture, 0.01 ml of 0.2 % *n*-valeric acid (internal standard in gas chromatography), 0.1 ml of ether and 1 drop of 6 N H_2SO_4 were added and *iso*-valeric acid was extracted into the ether. The separated ether layer was directly injected into a gas chromatography column (10 % polyethyleneglycol adipate plus 1 % H_3PO_4, chromosorb W HMDS, 120°C). A unit of pepstatin hydrolase is defined as the amount liberating one nanomole of *iso*-valeric acid per minute. The cells were harvested at after 55 hours growth and washed with 0.01 M phosphate buffer, pH 7.0, and frozen at −20°C as described in the previous paper.[3] The frozen cells maintained the activity for at least 3 months at this temperature. All subsequent operations for purification of the enzyme were carried out under 4°C except for the heat-step.

The frozen cells (1,850 g), thawed and suspended in 4,000 ml of 0.01 M phosphate buffer, pH 7.0, were disrupted by two passages through a French press (400 kg/cm^2). Cell debris was removed by centrifugation of the disrupted cell suspension at 10,000×g for 20 minutes. The cell-free extract thus obtained was heated at 60°C for 10 minutes. After

Fig. 1. Purification of pepstatin hydrolase by DEAE-cellulose chromatography

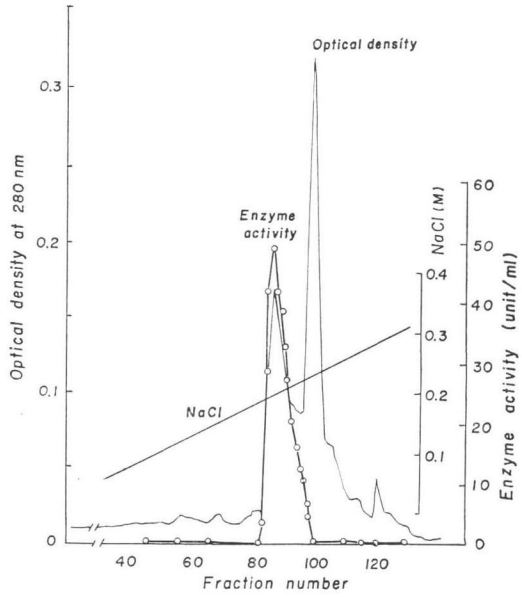


Fig. 2. Analytical disc gel electrophoresis of pepstatin hydrolase

Pepstatin hydrolase (70 μg of protein) was subjected to electrophoresis at pH 9.4 under the conditions described by DAVIS.[4] The gel was stained with Coomassie Brilliant Blue R.

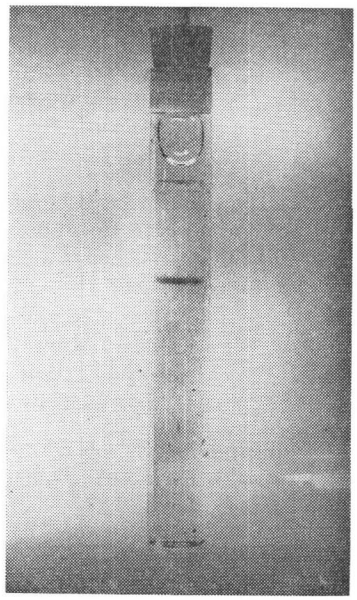


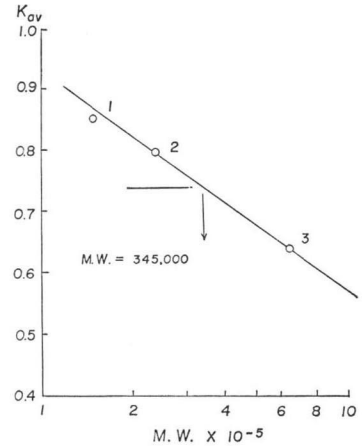
Table 1. Purification of pepstatin hydrolase from *Bacillus sphaericus*

Step	Volume (ml)	Total activity (unit)	Total protein (g)	Specific activity (unit/mg)	Yield (%)
Cell-free extract	4,700	38,740	1,549.6	0.025	100
Heated fraction	4,400	35,640	324.0	0.11	92
DEAE-cellulose pH 8.0	3,800	27,090	51.1	0.53	70
Sephadex G-200	225	20,310	0.278	73.0	52
DEAE-cellulose pH 7.0	430	12,734	0.082	155.1	33
DEAE-cellulose pH 6.5	102	7,169	0.032	225.0	19

removal of the precipitate by centrifugation, the supernatant was dialyzed against 18 liters of 0.01 M Tris-HCl buffer, pH 8.0, for 30 hours. A small amount of insoluble material which appeared during dialysis was removed by centrifugation and the dialyzed solution was loaded onto a 2,800 ml DEAE-cellulose column equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. The column was washed with 8,000 ml of the same buffer, and then with 4,000 ml of 0.3 M NaCl dissolved in the buffer. The active fractions eluted by 0.3 M NaCl were combined and concentrated by ultrafiltration and applied to a Sephadex G-200 colunm (6×90 cm) equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. Active fractions eluted from the column were pooled and dialyzed against 0.02 M phosphate buffer, pH 7.0. The dialyzed solution was applied to a DEAE-cellulose column (2.5×42 cm) equilibrated with the same buffer. The column was washed with 0.1M NaCl dissolved in the buffer, and the adsorbed enzyme was eluted with 2,200 ml linear gradient of NaCl from 0.1 to 0.3 M in the same buffer. Active fractions were combined and, after overnight dialysis against 0.02 M phosphate buffer, pH 6.5, were applied to a column of DEAE-cellulose (1.6×40 cm) equilibrated with the same buffer. An 800-ml linear gradient of NaCl from 0.1 to 0.3 M in the same buffer was used for elution of the enzyme (Fig. 1). Active fractions, pooled and concentrated by ultrafiltration, were dialyzed against 0.01 M phosphate buffer, pH 7.0 and stored at −20°C as a purified enzyme preparation. The purification and yields of the enzyme are summarized in Table 1.

The purified enzyme preparation demonstrated a single narrow band in disc electrophoresis on polyacrylamide gel at pH 9.4[4] (Fig. 2). The result of the gel filtration with Sepharose 4B[5] in 0.02 M phosphate buffer, pH 7.0, containing 0.15 M NaCl indicated a molecular weight of 345,000 as shown in Fig. 3. Ultracentrifugal sedimentation in a linear glycerol gradient[6] showed a single active peak at 14.5S. Electrophoresis on a acrylamide gel containing Ampholine (pH 3~10) as prepared by the method of DALE and LATNER,[7] showed a single band with pI 4.2. When the purified preparation was dissociated in 0.1 % sodium dodecylsulfate containing 1 % 2-mercaptoethanol and subjected to electrophoresis as described by WEBER and OSBORN,[8] a single band was observed. The migration of this band corresponded to a molecular weight of 45,500. When the molecular weight of the undissociated enzyme as described

Fig. 3. Molecular weight determination of purified pepstatin hydrolase on Sepharose 4B



The references used for calibration were human-γ-globulin (1), catalase (2) and thyroglobulin (3). Pepstatin hydrolase is indicated by arrows.

above, is compared to the smaller molecular weight of the dissociated enzyme, it suggests that the enzyme is constructed of subunits.

The enzyme showed maximum activity at 63°C and pH 7.0~7.5. It was stable in the pH range between 6.5 and 9.5, and completely active when exposed to 55°C for 10 minutes at pH 7.0. However, 40 % of the activity was lost after 10 minutes at 80°C and at 90°C for 10 minutes, all activity was lost. Metal-chelating agents such as EDTA and *o*-phenanthroline decreased the activity to 36 and 53 % of the original, respectively, at the concentration of 0.1 mM. Diisopropylfluorophosphate, an active serine-directed reagent, failed to cause inactivation at 10 mM. Typical sulfhydryl reagents such as $Hg(OCOCH_3)_2$ and *p*-chloromercuribenzoate at 2 mM, almost completely inhibited the enzyme. In addition, the SH-blocking agents, dithiothreitol and 2-mercaptoethanol at 2 mM also showed complete inhibition. Co^{++} and Ca^{++} at 10 mM stimulated the enzyme reaction 1.66 and 1.29-fold, respectively. The activity was reduced to 78~44 % of the original as the concentration of Zn^{++} increased from 0.05 to 10 mM.

The specificity of the enzme was determined using various pepstatins as the substrate (Table 2). All pepstatins tested (A, B[9] and Ac[10]) were hydrolyzed and gave their fatty acids, such as *iso*-valeric, *n*-caproic and acetic acids, together with valine and Val-AHMHA-Ala-AHMHA. Hydroxypepstatin A,[11] containing a serine residue instead of alanine in pepstatin A, was hydrolyzed and gave *iso*-valeric acid, valine and Val-AHMHA-Ser-AHMHA. Pepstanone A,[9] containing 3-amino-5-methylhexanone-2 group instead of

Table 2. Substrate specificity of pepstatin hydrolase

Substrates	Relative activity (%)	Ninhydrin-positive products
iso-Valeryl-Val-Val-AHMHA-Ala-AHMHA (pepstatin A)	100	Val, Val-AHMHA-Ala-AHMHA
n-Caproyl-Val-Val-AHMHA-Ala-AHMHA (pepstatin B)	723	Val, Val-AHMHA-Ala-AHMHA
Acetyl-Val-Val-AHMHA-Ala-AHMHA (pepstatin Ac)	250	Val, Val-AHMHA-Ala-AHMHA
iso-Valeryl-Val-Val-AHMHA-Ser-AHMHA (hydroxypepstatin A)	55	Val, Val-AHMHA-Ser-AHMHA
iso-Valeryl-Val-Val-AHMHA-Ala-3-amino-5-methylhexanone-2 (pepstanone A)	2	Val, Val-AHMHA-Ala-3-amino-5-methyl-hexanone-2
Acetyl-Val-AHMHA-Ala-AHMHA	0	None
iso-Valeryl-Val-AHMHA-Ala-AHMHA	0	None
Benzoyl-Val-AHMHA-Ala-AHMHA	0	None
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	0	None
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	0	None
iso-Valeryl-Val	0	None
iso-Valeryl-Val-Val	0	None
iso-Valeryl-Val-Val-AHMHA	55	Val, Val-AHMHA
iso-Valeryl-Val-Val-AHMHA-Ala-AHMHA	100	Val, Val-AHMHA-Ala-AHMHA
Val-Val-AHMHA		Val, Val-AHMHA

Activities were measured using each substrate according to the method described in the text. Relative activities were calculated based on the value obtained with pepstatin A. Hydrolysis of the substrates was also examined by thin-layer chromatography. A 0.01 ml portion of the hydrolysate was spotted on a silica gel plate with appropriate references and chromatographed using *n*-butanol-acetic acid-water (4:1:1).

Detection was carried out by ninhydrin reaction.

the C-terminal AHMHA in pepstatin A, was also hydrolyzed but less rapidly than pepstatin A. N-Acyl derivatives of the tetrapeptide which was obtained by the enzymatic hydrolysis of pepstatin A (R-Val-AHMHA-Ala-AHMHA),[12] were insusceptible to this enzyme, indicating that the presence of two valine moieties in pepstatins is necessary to undergo this enzyme reaction.

The action of the enzyme on a series of N-*iso*-valeryl peptides,[13] *i.e. iso*-valeryl-valine, *iso*-valeryl-Val-Val and *iso*-valeryl-Val-Val-AHMHA, reveals that *iso*-valeryl-Val-Val-AHMHA or R-Val-Val-AHMHA is the shortest chain length to become the substrate for this enzyme, because neither *iso*-valeryl-valine nor *iso*-valeryl-Val-Val was hydrolyzed, but *iso*-valeryl-Val-Val-AHMHA was susceptible to this enzyme. From the result that *iso*-valeryl-Val could not be served as the substrate but Val-Val-AHMHA was broken down to valine and Val-AHMHA as rapidly as pepstatins, we can presume that pepstatins are first deacylated and secondly, Val-Val-AHMHA-Ala-AHMHA thus produced is further hydrolyzed to give valine and Val-AHMHA-Ala-AHMHA. Further studies on this interesting hydrolase are under investigation.

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