Affinity Chromatography of Neutral Proteinase from *Streptomyces naraensis* on a Column of *N*-Carbobenzoxy-L-phenylalanyl-aminoethyl-Sepharose†

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N-Carbobenzoxy-L-phenylalanyl-aminoethyl-Sepharose (Z-Phe-AH-Sepharose) was found to be an effective affinity adsorbent for neutral proteinase from *Streptomyces naraensis*. This enzyme was adsorbed on this affinity column at pH 7.0 or higher, and eluted with 0.3 M NaCl in starting buffer. The resulting enzyme appeared homogeneous on disc electrophoresis and showed considerably higher specific activity and recovery than by previous method.

In the previous studies of this series,^{1~4)} a neutral proteinase [EC 3.4.24 group] was purified from the culture media of *Streptomyces naraensis* to an electrophoretically homogeneous state by fractionation with acetone, gel filtration on Sephadex G-100 and column chromatography on DEAE-cellulose. The enzymatic¹⁾ and protein chemical properties³⁾ of this enzyme, containing 1 g-atom of zinc per mol of enzyme protein⁴⁾ (based on a molecular weight of 37,000), were reported by the author and Ouchi.

Affinity chromatography of metalloenzymes, which are common among neutral proteolytic enzymes (endopeptidases) from microorganisms, has been reported by some investigators: neutral proteinase from *Clostridium histolyticum* by Sparrow and McQuade,⁵⁾ neutral subtilopeptidase *amylosacchariticus* and thermolysin from *Bacillus* species by Fujiwara *et al.*,⁶⁾ and Komiyama *et al.*⁷⁾

N-Carbobenzoxy-L-phenylalanyl-aminoethyl-Sepharose was found to be an effective affinity adsorbent for purifying neutral proteinase from *Streptomyces naraensis* by

affinity chromatography.

On the other hand, inhibition by carbobenzoxy-amino acid and peptides on the activity of neutral subtilopeptidase was reported by Morihara *et al.*⁸⁾

The activity of neutral proteinase from *Streptomyces naraensis* was inhibited by carbobenzoxy amino acids (Z-Phe-OH, and Z-Leu-OH*¹), which were found to be competitive inhibitors. The inhibition by carbobenzoxy amino acids of the enzyme will be reported in the subsequent paper.*²

The present paper reported that the neutral proteinase from *Streptomyces naraensis* can be purified by affinity chromatography, using a column of *N*-carbobenzoxy-L-phenylalanyl-aminoethyl-Sepharose (Z-Phe-AH-Sepharose).

MATERIALS AND METHODS

Chemicals. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) was purchased from Nakarai Chemical Co., carbobenzoxy amino acids such as Z-Phe-OH and Z-Leu-OH were obtained from the Peptide Institute, Protein Research Foun-

† Neutral Proteinase from *Streptomyces naraensis*. Part V. For Parts I~IV, see ref. 1~4.

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*¹ Abbreviations: Z-Phe-OH, carbobenzoxy-L-phenylalanine, Z-Leu-OH, carbobenzoxy-L-leucine.

*² Neutral Proteinase from *Streptomyces naraensis*. Part VII.

dation, and *N*-aminohexylamino-Sepharose 4B (AH-Sepharose) was a product of Pharmacia Fine Chemicals Co.

Enzyme preparation. The neutral proteinase from *Streptomyces naraensis* chromatographed on a column of Sephadex G-100 was prepared by the method described previously.³⁾ The specific activity of the gel filtrated enzyme was 1271 mkat per kg of protein.

Assay for proteinase activity. Proteinase activity was measured by the casein-275 nm method of Hagiwara *et al.*⁹⁾ as described previously.¹⁾

Assay for protein concentration. The protein concentration of each fraction was measured at 280 nm using $E_{1\text{ cm}}^{1\%} = 11.22$ or by Lowry *et al.*'s¹⁰⁾ method as described previously.¹⁾

Zinc analysis. Zinc was determined by atomic absorption spectrophotometry as described previously.⁴⁾

Disc electrophoresis. Disc electrophoresis of the enzyme was performed by the method of Ornstein¹¹⁾ and Davis¹²⁾ as described previously.³⁾

Preparation of *N*-carbobenzoxy-L-phenylalanyl- (Z-Phe-AH-Sepharose) and *N*-carbobenzoxy-L-leucyl-aminohexyl-Sepharose (Z-Leu-AH-Sepharose). Five g of *N*-aminohexylamino-Sepharose 4B (AH-Sepharose) washed with 0.5 M NaCl and deionized water was mixed with 24 mg of CMC and 150 mg of Z-Phe-OH or Z-Leu-OH in 20 ml of 40% *N,N*-dimethylformamide solution at pH 5.0, and then left at room temperature with gentle shaking for one day. After the reaction, the gels were washed successively with 1 M NaCl containing 40% *N,N*-dimethylformamide, 1 M NaHCO₃, deionized water, 1 M acetic acid and finally with deionized water, and were then stored in the cold until use. These Z-Phe-AH- and Z-Leu-AH-Sepharoses were found to contain 6 μmol of phenylalanine and 10 μmol of leucine per g of dried gel, respectively.

Affinity chromatography. The gel filtrated enzyme solution was chromatographed on columns (0.5 $\times$ 1.5 cm) of Z-Phe-AH-, Z-Leu-AH-, or AH-Sepharose, which had been equilibrated with 0.033 M^{*} veronal-sodium acetate-HCl buffer (Michaelis) at various pHs containing 0.01 M calcium acetate (ionic strength, $\mu = 0.05$). The enzyme was eluted with linearly increasing concentrations of NaCl in starting buffer, at a flow rate of 19.5 ml per hr, and fractions of 1.7 ml were collected. The liquid from each tube was subjected to measurement for enzymatic activity and protein concentration.

*³ The concentration of buffer solution is expressed in mol of veronal.

RESULTS

Effect of pH on adsorption of the neutral proteinase on the affinity chromatography

The effect of pH on adsorption of the enzyme on the affinity chromatography using columns of Z-Phe-AH-, Z-Leu-AH-, or AH-Sepharose, which had been equilibrated with 0.033 M veronal-sodium acetate-HCl buffer at various pHs containing 0.01 M calcium acetate was studied.

The enzyme was adsorbed on columns of Z-Phe-AH- or Z-Leu-AH-Sepharose at pH 7.0 or higher. But this enzyme was not adsorbed on columns of AH-Sepharose at pH 7.2 or lower, or of Z-Phe-AH- or Z-Leu-AH-Sepharose at pH 6.5, respectively. Elution profiles of the neutral proteinase on columns of Z-Phe-AH-, Z-Leu-AH-, or AH-Sepharose are shown in Fig. 1. The enzyme adsorbed on columns of Z-Phe-AH- or Z-Leu-AH-Sepharose was eluted with 0.3 M NaCl in the

FIG. 1. Elution Profiles of Neutral Proteinase.

The procedure was given in MATERIALS AND METHODS except that 0.033 M veronal-sodium acetate-HCl buffer, pH 7.0, containing 0.01 M calcium acetate was used. The columns used were Z-Phe-AH- (A), Z-Leu-AH- (B), and AH-Sepharose (C), respectively, —, ultraviolet absorption at 280 nm; —○—, proteolytic activity; ---, concentration of NaCl.

starting buffer at pH 7.0 for Z-Phe-AH-Sepharose and with 0.2 M NaCl in the same buffer for Z-Leu-AH-Sepharose, respectively.

Improved purification using affinity chromatography

A neutral proteinase was purified by an improved method using gel filtration on Sephadex G-100 and column chromatography on Z-Phe-AH-Sepharose.

The neutral proteinase purified on the col-

FIG. 2. Affinity Chromatography of Neutral Proteinase. Enzyme solution (16 ml) fractionated by chromatography on Sephadex G-100 was placed on the top of a column (1.0 × 6.2 cm) of Z-Phe-AH-Sepharose which had been equilibrated with 0.025 M Tris-HCl buffer, pH 7.2, containing 0.01 M calcium acetate. The enzyme was eluted with linearly increasing concentrations of NaCl from 0 to 0.7 M in the starting buffer. The flow rate was 26.6 ml per hr and fractions of 5 ml were collected. The horizontal arrow indicates fractions pooled. —, ultraviolet absorption at 280 nm; —○—, proteolytic activity; ---×---, specific activity; ----, concentration of NaCl.

umn of Sephadex G-100 was applied on a column (1.0 × 6.2 cm) of Z-Phe-AH-Sepharose which had been equilibrated with 0.025 M Tris-HCl buffer at pH 7.2 containing 0.01 M calcium acetate. After the column was washed with the starting buffer, the enzyme was eluted with linearly increasing concentrations of NaCl in the starting buffer, at a flow rate of 26.6 ml of per hr, and fractions of 5 ml were collected. As shown in Fig. 2, after elution with 150 ml of the starting buffer, a reservoir containing the second buffer (0.025 M Tris-HCl buffer at pH 7.2 containing 0.01 M calcium acetate and 0.7 M sodium chloride, 150 ml) was connected to the mixing chamber filled with 150 ml of the starting buffer.

For further purification, the fractions indicated by the horizontal arrow in Fig. 2 containing enzyme were combined and dialyzed against 0.005 M calcium acetate at pH 7.5.

The results of the purification procedures are summarized in Table I. The specific activity of this highly purified neutral proteinase was 3212 mkat per kg of protein, and 12 mg of enzyme protein was obtained from 330 ml of culture media with a yield of 63 per cent.

On acrylamide gel electrophoresis, the purified enzyme migrated to the anode as a single band.

This purified enzyme was confirmed to contain 1.0 g-atom of zinc per mol of enzyme (2.75×10^{-8} mol of zinc per mg of protein),

TABLE I. PURIFICATION OF NEUTRAL PROTEINASE FROM *Streptomyces naraensis*

One unit of the proteolytic activity was defined as the amount of enzyme that liberated trichloroacetic acid soluble hydrolysate from casein corresponding to one μ mol of L-tyrosine per min per ml of enzyme solution at 40°C.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (mkat/kg)	Recovery (%)
Crude enzyme solution	3,044	3,653	20	100
Acetone precipitation	213	3,288	258	90
Sephadex G-100 chromatography	37	2,815	1,271	77
Z-Phe-AH-Sepharose affinity chromatography	12	2,308	3,212	63

based on a molecular weight of 37,000 and from the zinc content determined by the atomic absorption spectrophotometric method.

DISCUSSION

Sparrow and McQuade⁵⁾ reported that the neutral proteinase from *Clostridium histolyticum* was adsorbed on a column of Sepharoyl-caproyl-glycyl-L-leucine (Sepharoyl-Cap-Gly-Leu-OH), equilibrated with the starting buffer at pH 7.2, and eluted with 0.05 M Tris at pH 10.

Fujiwara *et al.*⁶⁾ reported that neutral subtilopeptidase and thermolysin were adsorbed on a column of carbobenzoxy-L-phenylalanyl-triethylenetetraaminyl-Sepharose (Z-Phe-T-Sepharose), equilibrated with the starting buffer at pH 7.2, and eluted with 0.05 M borate buffer containing 0.5 M NaCl at pH 9.0 for neutral subtilopeptidase and 1.5 M guanidine in the starting buffer at pH 7.2 for thermolysin, respectively.

Komiyama *et al.*⁷⁾ reported that neutral subtilopeptidase and thermolysin were adsorbed on a column of phosphoramidon-Sepharose, equilibrated with the starting buffer at pH 7.0, and eluted with 1.0 M NaCl in 0.1 M Tris-HCl buffer at pH 9.0 for neutral subtilopeptidase, and 1.0 M guanidine-HCl in 0.1 M Tris-HCl buffer at pH 9.0 for thermolysin, respectively.

The author confirmed that the neutral proteinase from *Streptomyces naraensis* was not adsorbed on a column of Sepharoyl-Cap-Gly-Leu-OH when adjusted at pH 7.2. The neutral proteinase is highly purified by affinity chro-

matography, when adsorbed on a column of Z-Phe-AH-Sepharose at neutral pH and eluted successfully with 0.3 M NaCl in the starting buffer (Fig. 3). The enzyme was isolated by the improved purification method with a yield of about 63 per cent, considerably higher than by previous method.³⁾ This purified enzyme is homogeneous on disc electrophoresis and contains 1 g-atom of zinc per mol of enzyme protein.

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REFERENCES

- 1) A. Hiramatsu, *J. Biochem.*, **62**, 353 (1967).
- 2) A. Hiramatsu, *J. Biochem.*, **62**, 364 (1967).
- 3) A. Hiramatsu and T. Ouchi, *J. Biochem.*, **71**, 767 (1972).
- 4) A. Hiramatsu and T. Ouchi, *Agric. Biol. Chem.*, **42**, 1309 (1978).
- 5) L. G. Sparrow and A. B. McQuade, *Biochim. Biophys. Acta*, **302**, 90 (1973).
- 6) K. Fujiwara, K. Osue and D. Tsuru, *J. Biochem.*, **77**, 739 (1975).
- 7) T. Komiyama, T. Aoyama, T. Takeuchi and H. Umezawa, *Biochem. Biophys. Res. Commun.*, **65**, 352 (1975).
- 8) K. Morihara, T. Oka and H. Tsuzuki, *Arch. Biochem. Biophys.*, **132**, 480 (1968).
- 9) B. Hagiwara, N. Matsubara, J. Nakai and K. Okunuki, *J. Biochem.*, **45**, 185 (1957).
- 10) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 11) L. Ornstein, *Ann. New York Acad. Sci.*, **121**, 321 (1964).
- 12) B. J. Davis, *Ann. New York Acad. Sci.*, **121**, 406 (1964).