# INHIBITORY EFFECTS OF PHOSPHORAMIDON ON NEUTRAL METALLOENDOPEPTIDASES AND ITS APPLICATION ON AFFINITY CHROMATOGRAPHY

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Summary: Phosphoramidon, a thermolysin inhibitor isolated from a culture filtrate of streptomyces, inhibited the other two neutral metalloendopeptidases [EC 3.4.24.4 group], and was shown to be a suitable ligand for affinity chromatography of these metalloendopeptidases.

Phosphoramidon, N-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, has been isolated from a culture filtrate of Streptomyces tanashiensis (MD706-Y4) (1, 2, 3) and is a powerful inhibitor of thermolysin. It does not inhibit the other endopeptidases such as trypsin, papain, chymotrypsin and pepsin (1, 2, 4).

Thermolysin is obtained from the culture filtrate of Bacillus thermoproteolyticus and belongs to a group of neutral metalloendopeptidases [EC 3.4.24.4 group]. The characteristics of this enzyme group are their sensitivity to chelating agents and their optimum pH near neutral (5).

In this paper action of phosphoramidon on the other neutral metalloendopeptidases and use of phosphoramidon as a ligand for affinity chromatography of these enzyme are reported.

## Materials and Methods

Crystalline thermolysin was purchased from Daiwa Kasei K. K., Osaka, Japan. Neutral subtilopeptidase from Bacillus subtilis var. amylosacchariticus was a kind gift from Dr. Daisuke Tsuru. The metalloendopeptidase of Streptomyces griseus K-1 was partially purified from pronase (Kaken Kagaku K. K., Tokyo) by application of the method of Morihara et al. (6). Phosphor-

amidon and N-phosphoryl-L-leucyl-L-tryptophan were prepared as described previously (1, 4). Carbobenzoxy-glycyl-L-leucineamide was purchased from Protein Research Foundation, Osaka. Casein from milk was obtained from Wako Pure Chemical Industries, Ltd., Osaka. AH-Sepharose 4B and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Pharmacia and Sigma, respectively. The enzyme activity of thermolysin was measured as described previously (4), and the activity of the other two neutral metalloendopeptidases, was measured by the same method, except that calcium chloride and sodium chloride were not added to the buffer solution. Mode of inhibition and Ki values were determined according to the method of Henderson plot (7).

Preparation of phosphoramidon-Sepharose 4B. One g of AH-Sepharose 4B washed with 0.5 M NaCl and with water was mixed with 77 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride and 53 mg of phosphoramidon in 5.0 ml of aqueous solution at pH 5.0, and left at room temperature with gentle stirring for one day. After reaction, the gel was packed in a small

Table 1

Inhibitory Effects of Phosphoramidon and Its Analogue on Neutral Metalloendopeptidases

| Compound | B.thermoproteolyticus | | S.subtilis var. amylosaccharitiicus | | S.griseus K-1 | |
|---|---|---|---|---|---|---|
| | Z-Gly-LeuNH$_2$ | Casein | Z-Gly-LeuNH$_2$ | Casein | Z-Gly-LeuNH$_2$ | Casein |
| | $ID_{50}$ (μg/ml) | | | | | |
| P-Leu-Trp | 0.007 | 0.018 | 0.2 | 0.15 | 0.002 | 0.029 |
| Rha-P-Leu-Trp | 0.04 | 0.36 | 4.7 | 19 | 0.06 | 1.1 |

$ID_{50}$: inhibitor concentration need for 50% inhibition of enzyme activity.
P-Leu-Trp: N-phosphoryl-L-leucyl-L-tryptophan,
Rha-P-Leu-Trp (Phosphoramidon): N-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan,
Z-Gly-LeuNH$_2$: carbobenzoxy-glycyl-L-leucineamide

column (1 x 5 cm), and washed successively with each 10 ml of the following solutions, 1 M NaCl, 0.1 M Tris-HCl containing 1 M NaCl, pH 8.0, 50 mM formic acid containing 1 M NaCl, pH 3.0, 0.1 M Tris-HCl containing 1 M NaCl, pH 8.0, 50 mM Tris-HCl, pH 8.0, and then equilibrated with 10 mM Tris-HCl containing 5 mM $CaCl_2$, pH 7.0.

The amount of uncoupled phosphoramidon was estimated by determining the absorbance at 281 nm of the washing solution (its molecular extinction coefficient is $4.54 \times 10^3$ $M^{-1}cm^{-1}$). Under this condition, about 10 umoles of the inhibitor was coupled with one ml of swelled gel.

## Results and Discussion

As shown in Table 1, phosphoramidon and N-phosphoryl-L-leucyl-L-tryptophan inhibited neutral metalloendopeptidases of three different microbial origins, B. thermoproteolyticus, B. subtilis var. amylosaccharitícus and S. griseus K-1. In all cases, N-phosphoryl-L-leucyl-L-tryptophan showed stronger inhibition than phosphoramidon, as reported previously on thermolysin (4).

Kinetic studies on these inhibitors were carried out using carbobenzoxy-glycyl-L-leucineamide as substrate (Table 2). All of these inhibitors inhibited

Table 2

Ki Values of Neutral Metalloendopeptidases

| Compound | Inhibition mode | Ki (M) | | |
|---|---|---|---|---|
| | | B. thermoproteolyticus | B. subtilis var. amylosaccharitícus | S. griseus K-1 |
| P-Leu-Trp | competitive | $2.0 \times 10^{-9}$ | $3.0 \times 10^{-7}$ | $1.1 \times 10^{-9}$ |
| Rha-P-Leu-Trp | competitive | $2.8 \times 10^{-8}$ | $6.5 \times 10^{-6}$ | $2.0 \times 10^{-8}$ |

Abbreviations are same in Table 1.

the enzymes in a competitive manner, and Ki values were in the order of $10^{-6}$ M to $10^{-9}$ M. N-phosphoryl-L-leucyl-L-tryptophan gave Ki values one order lower than that of phosphoramidon.

Phosphoramidon was coupled with Sepharose 4B and affinity chromatography of thermolysin was carried out on phosphoramidon-Sepharose column (Fig. 1). Thermolysin was not eluted from the column with 0.1 M Tris-HCl containing 5 mM $CaCl_2$, 1.0 M guanidine hydrochloride, pH 7.0, but it was eluted when pH of the buffer was raised to 9.0. The yield of activity was 88% and specific activity was raised by 30%. Similarly neutral subtilopeptidase from B. subtilis var. amylosaccharitícus was applied on phosphoramidon-Sepharose 4B column (Fig. 2). Again, the enzyme was eluted when pH of the buffer was changed from 7.0 to 9.0. A 100% recovery was achieved.

Except metal chelating reagents (5), only few compounds have been

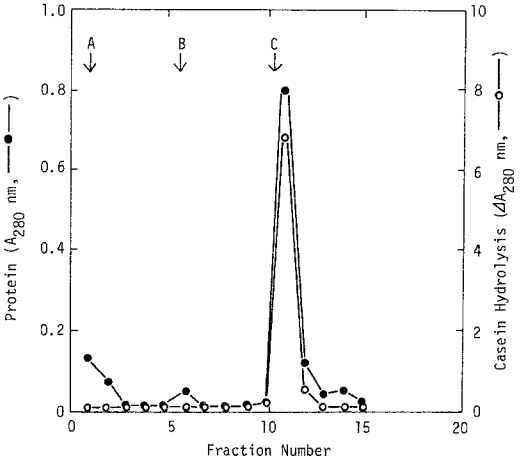


Fig. 1. Affinity chromatography of thermolysin. The column (0.4 x 2.0 cm) was first equilibrated with 10 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.0. The enzyme (1.5 mg protein) in the same buffer was applied and fractions of 1.5 ml were collected. The elution was performed with the following solutions, A: 10 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.0. B: 0.1 M Tris-HCl, 5 mM $CaCl_2$, 1.0 M guanidine hydrochloride, pH 7.0. C: same as B except that pH was adjusted to 9.0.
The enzyme activity was expressed as the absorbance change due to casein hydrolysis with 20 µl of each fraction.

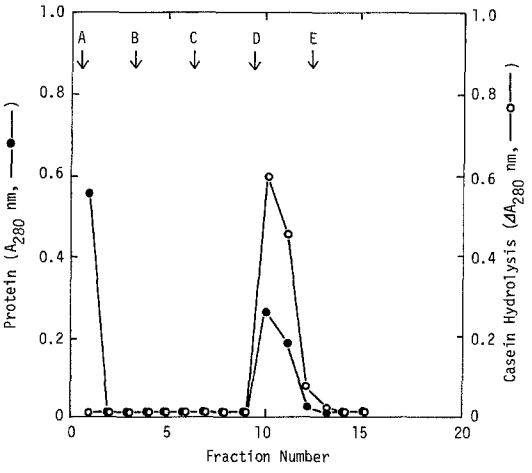


Fig. 2. Affinity chromatography of neutral subtilopeptidase. The column (0.4 x 2.0 cm) was first equilibrated with 10 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.0. The enzyme from B. subtilis var. amylosaccharitícus (3.0 mg protein) in the same buffer was applied and fractions of 3.0 ml were collected. The elution was performed with the following solutions, A: 10 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.0. B: 0.1 M Tris-HCl, 5 mM $CaCl_2$, pH 7.0. C: same as B except containing 1.0 M NaCl. D: same as B except that pH was adjusted to 9.0. E: same as B except containing 1.0 M NaCl at pH 9.0. The enzyme activity was expressed as absorbance change using 3 µl of each fraction.

reported to inhibit neutral metalloendopeptidases. Morihara et al. (8) reported D-amino acid containing carbobenzoxy peptide as a competitive inhibitor of the enzyme from B. subtilis var. amylosaccharitícus. J. Feder et al. (9) reported dipeptide or carbobenzoxy dipeptide as a competitive inhibitor of thermolysin. In both cases, the action was very weak and Ki values were in the order of $10^{-4}$ M to $10^{-1}$ M. However, phosphoramidon which has a unique structure does not show any inhibition on serine, sulfhydryl and acid endopeptidases but it shows strong inhibition against three neutral metalloendopeptidases. As shown in Table 2, Ki values of phosphoramidon are in the order of $10^{-6}$ M to $10^{-8}$ M. Moreover, N-phosphoryl-L-leucyl-L-tryptophan shows stronger inhibition with lower Ki values in the order of $10^{-7}$ M to $10^{-9}$ M. These results suggest that phosphoramidon and the N-phosphoryl peptide should be a general inhibitor of neutral metalloendopeptidases and that useful in

studying structure and function of the active sites of these enzymes.

According to the substrate specificity of neutral metalloendopeptidases, it is suggested that L-leucyl-L-tryptophan moiety of phosphoramidon may interact with the substrate binding site of the enzyme. Furthermore, as described in a previous paper (4), N-phosphate moiety in phosphoramidon was shown to be essential for the action. Thus, N-phosphate of L-leucyl-L-tryptophan shows strong inhibition.

The inhibitors with a strong action should be useful as ligands for affinity chromatography. Although other type of ligands has been already reported (8, 9), phosphoramidon seems to be a more suitable ligand which can give efficient purification, as shown in Fig. 1 and 2.

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