Purification and characterization of ascamycin-hydrolysing aminopeptidase from *Xanthomonas citri*

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A nucleoside antibiotic, ascamycin {9- β -[5'-O-(N-L-alanyl)sulphamoyl-D-ribofuranosyl]-2-chloroadenine}, has a selective antibacterial activity against *Xanthomonas* species. When ascamycin was dealanylated, dealanylascamycin showed a broad antibacterial activity against various Gram-negative and Gram-positive bacteria. *Xanthomonas citri* is susceptible to ascamycin by virtue of the ascamycin-dealanylating enzyme on the cell surface [Osada & Isono (1985) *Antimicrob. Agents Chemother.* **27**, 230–233]. The enzyme (Xc aminopeptidase) was purified from *X. citri* cells by successive DEAE-cellulose, chromatofocusing and Sephadex G-100 column chromatography to a homogeneous state. The purified enzyme exhibited a single band with an M_r of 38 000 in SDS/polyacrylamide-gel electrophoresis. Gel filtration on a calibrated column indicated a similar M_r value. The isoelectric point of the enzyme was 5.7. The enzyme catalysed the hydrolysis of the alanyl group of ascamycin and liberated alanine from the sulphamoyl nucleoside. The enzyme also catalysed the hydrolysis of L-proline β -naphthylamide and L-alanine β -naphthylamide. The optimal pH and temperature for enzyme activity were pH 7.5–8.0 and 35–40 °C respectively. The enzyme was inhibited by thiol-enzyme inhibitors (i.e. *p*-chloromercuribenzoate and *N*-ethylmaleimide), but was not affected by various naturally occurring aminopeptidase inhibitors (i.e. amastatin, bestatin, pepstatin and leupeptin). Mn^{2+} and Mg^{2+} activated the enzyme, whereas Cu^{2+} , Zn^{2+} and Cd^{2+} were inhibitory.

INTRODUCTION

The nucleoside antibiotic ascamycin has a selective toxicity to plant pathogenic *Xanthomonas* species, whereas dealanylascamycin shows a broad antibacterial activity (Isono *et al.*, 1984). We have shown (Osada & Isono, 1985) that both ascamycin and dealanylascamycin inhibit strongly protein synthesis *in vitro* but that the bactericidal activity of ascamycin is masked by the alanyl group which interferes with membrane transport in bacteria. *Xanthomonas citri* is susceptible to ascamycin by virtue of an ascamycin-hydrolysing enzyme (Scheme 1) that exists on the cell surface. Other bacteria, which lack the enzyme on the cell surface, are not susceptible to ascamycin. To elucidate the properties and the physiological role of the enzyme and the distribution of the enzyme in various prokaryotic and eukaryotic cells, we attempted the isolation of the enzyme.

The present paper concerns with purification and characterization of the ascamycin-dealanylating enzyme from *X. citri*. The enzyme was found to be specific to prolyl- and alanyl-peptides and was designated as Xc aminopeptidase.

EXPERIMENTAL

Strain and growth condition

The plant pathogenic bacterium *Xanthomonas citri* I.F.O. 3781 was obtained from the Institute for Fermentation, Osaka, Japan. The organism was grown aerobically in medium containing 18 g of nutrient broth (Eiken, Tokyo, Japan), 6 g of dried yeast (Daigo, Tokyo, Japan), 6 g of malt extract (Difco Laboratories, Detroit,

MI, U.S.A.) and 10 g of glycerol per litre of potato-extract solution at 28 °C.

Chemicals

Amino acids β -naphthylamides (those of glycine, L-alanine, DL-alanine, L-valine, L-leucine, L-arginine,

Scheme 1. Enzymic dealanylation of ascamycin

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L-proline, L-histidine, L-methionine, L-serine, L-glutamine and L-isoleucine) were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phenylmethanesulphonyl fluoride and L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK') were also the products of Sigma Chemical Co. *N*-Ethylmaleimide and *p*-chloromercuribenzoate were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Aminopeptidase inhibitors (amastatin, bestatin, pepstatin and leupeptin) were purchased from Protein Research Foundation (Osaka, Japan).

Assay of enzyme activity

A 10 μ l portion of the enzyme solution was mixed with 20 μ g of ascamycin in 20 mM-Tris/HCl buffer, pH 7.8, and incubated at 37 °C for 90 min. The reaction mixture was analysed by silica-gel t.l.c. [Merck Kiesegel 60F₂₅₄, solvent system propan-2-ol/1 M-NH₃ (7:3, v/v)]. The dealanylation activity was detected by the alteration in R_F value of u.v.-detectable spots [from ascamycin (0.59) to dealanylascamycin (0.72)] and the appearance of the antibacterial activity against *Escherichia coli* BE1186 (Otsuji *et al.*, 1978). Enzyme activity can be measured semi-quantitatively by the paper-disc/agar-plate method.

Spectrometric measurement of enzyme activity

In a cuvette, 0.4 ml of 20 mM-Tris/HCl buffer, pH 7.8, and 0.5 ml of 2 mM-L-alanine β -naphthylamide (dissolved in the same buffer) were placed and left to equilibrate at 37 °C. At zero time, 0.1 ml of the test enzyme solution was added to the cuvette and the contents were mixed. For enzyme inhibition experiments, an enzyme concentration of 50 μ g/ml was used. The changes in A_{340} were directly recorded with a Hitachi model 22A spectrophotometer. In these conditions, the rate of hydrolysis of L-alanine β -naphthylamide was calculated by using $\Delta\epsilon = 1.78 \times 10^3$ M⁻¹·cm⁻¹ (Lee *et al.*, 1971).

Protein determination

Protein was measured by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A), with ovalbumin (grade V; Sigma Chemical Co.) as standard. In all chromatographic procedures the protein concentration was determined by measurement of the absorbance at 280 nm.

Enzyme purification

Extraction. Xc aminopeptidase was extracted from 242 g (wet wt.) of *X. citri* cells with 600 ml of extraction buffer (0.2% sodium deoxycholate in 10 mM-Tris/HCl buffer, pH 7.8).

Protamine sulphate precipitation. Protamine sulphate (Wako Pure Chemical Industries, Osaka, Japan) was added to the extracted solution to a final concentration of 0.2% to precipitate nucleic acids.

Ammonium sulphate precipitation. After the precipitate had been removed by centrifugation, to the supernatant was added solid (NH₄)₂SO₄ to give a final concentration of 40% saturation. The mixture was centrifuged and the precipitate discarded. To the supernatant solution was added solid (NH₄)₂SO₄ to give a final concentration of 60% saturation. The precipitate collected by centrifugation was dissolved in 100 ml of the standard buffer (10 mM-Tris/HCl buffer, pH 7.8) and the solution was dialysed against the same buffer.

DEAE-cellulose chromatography. The sample was applied on a DEAE-cellulose (DE52; Whatman, Maidstone, Kent, U.K.) column (4 cm $\times$ 20 cm) previously equilibrated with the standard buffer and eluted with a linear gradient of 0–1 M-KCl in the same buffer. Xc aminopeptidase was eluted at 450–550 mM-KCl. Active fractions were pooled and concentrated with a Diaflo membrane PM10 (Amicon Co., Lexington, MA, USA.).

Chromatofocusing chromatography. The concentrated solution was dialysed against 25 mM-imidazole/HCl buffer pH 7.4, and applied on to a chromatofocusing (PBE74; Pharmacia Co., Uppsala, Sweden) column (1 cm $\times$ 7 cm) previously equilibrated with the same buffer. The sample was eluted with PB74 (Pharmacia)/HCl buffer, pH 4.0. Xc aminopeptidase was eluted at pH 5.7–5.3.

Gel filtration. Active fractions were pooled, concentrated with a Diaflo membrane PM10 and applied on to a Sephadex G-100 (Pharmacia) column (1.6 cm $\times$ 65 cm) previously equilibrated with TN buffer (20 mM-Tris/HCl buffer, pH 7.8, containing 100 mM-NaCl). Active fractions, which emerged at $V_e/V_0 = 1.75$, were pooled and concentrated by means of dialysis against 20% (v/v) glycerol in TN buffer. This sample was stored at –20 °C.

Measurement of M_r

The M_r of the purified enzyme was determined by gel filtration on a Sephadex G-100 column (1.6 cm $\times$ 65 cm) by the method of Andrews (1965), with TN buffer. SDS/12% polyacrylamide-gel electrophoresis as described by Laemmli (1970) was also used for measurement of the M_r of the enzyme.

Isoelectric point

Isoelectric-focusing electrophoresis was performed with a Pharmacia FBE3000 flat-bed apparatus at 5 °C, with 5% acrylamide gel containing Pharmalyte (pH 4.0–6.5). The isoelectric point was calibrated by the use of a kit of standard proteins (Pharmacia).

RESULTS

Purification of Xc aminopeptidase

Because Xc aminopeptidase is on the cell envelope, one of the most critical steps in the purification of this enzyme is the solubilization of the enzyme activity from the cell. A 10 mM-Tris/HCl buffer, pH 7.8, containing 0.2% sodium deoxycholate was used for extraction. The crude extract was treated with 0.2% protamine sulphate to remove nucleic acids.

After fractionation with (NH₄)₂SO₄ of 40–60% saturation, the crude enzyme solution was applied on to a DEAE-cellulose column and eluted with a linear gradient of KCl. Alanine-liberating activity with L-alanine β -naphthylamide as substrate was detected both in the passed solution and in the 450–550 mM-KCl eluate. However, only the latter fraction showed the ascamycin-dealanylation activity (Fig. 1), and it was further purified by chromatofocusing (Fig. 2) and Sephadex G-100 column chromatography. The procedures and results for purification of Xc aminopeptidase are summarized in Table 1.

Fig. 1. Elution profile of Xc aminopeptidase from a DEAE-cellulose column

For experimental details see the text. —, A_{280} ; ---, KCl gradient; ●, ascamycin-dealanylation activity, indicated by inhibition diameter, with paper disc of 8 mm diameter.

Fig. 2. Elution profile of Xc aminopeptidase from a chromatofocusing column

For experimental details see the text. —, A_{280} ; ○, pH gradient; ●, enzyme activity with alanine β -naphthylamide as the substrate.

Determination of M_r and isoelectric point

The M_r of the purified enzyme was determined to be 38000 by gel filtration and SDS/polyacrylamide-gel electrophoresis (Fig 3a), suggesting that the Xc aminopeptidase is a monomer. The isoelectric point was 5.7, as measured by the isoelectric-focusing electrophoresis (Fig. 3b).

Fig. 3. SDS/polyacrylamide-gel electrophoresis (a) and isoelectrofocusing electrophoresis (b) of purified Xc aminopeptidase

For experimental details see the text. (a) SDS/polyacrylamide-gel electrophoresis. The marker proteins are as follows: phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20100) and α -lactalbumin (M_r 14400). (b) Isoelectric-focusing electrophoresis. The marker proteins are β -lactalbumin A (pI 5.20) and bovine carbonic anhydrase B (pI 5.85).

Substrate specificity

Xc aminopeptidase hydrolysed ascamycin to give L-alanine and dealanylascamycin, and also catalysed the hydrolysis of peptides (L-alanyl-L-alanine and glycyl-glycine). D-Alanyl-D-alanine was not hydrolysed. When V_{max}/K_m ratios of the enzyme against several substrates were compared, the enzyme was shown to liberate

Table 1. Purification of Xc aminopeptidase

Experimental details are given in the text. One unit of activity can catalyse the hydrolysis of 1 μ mol of L-alanine β -naphthylamide/min at 37 °C.

Isolation step	Protein (mg)	Activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (fold)
Extraction	965	168	100	0.17	1.0
Protamine	980	165	98.2	0.17	1.0
(NH ₄) ₂ SO ₄	365	143	85.1	0.39	2.3
DEAE-cellulose	16.5	86	51.2	5.2	31
Chromatofocusing	2.5	63	37.5	25.2	148
Sephadex G-100	1.1	59	35.1	53.6	315

Table 2. Substrate specificity of Xc aminopeptidase

For experimental details see the text. Abbreviation: β -Nap, β -naphthylamide. —, Not hydrolysed.

Substrate	K_m (mM)	$V_{max.}$ (μ mol/min per mg)	$V_{max.}/K_m$
L-Ala- β -Nap	2.1	56.0	26.7
DL-Ala- β -Nap	4.0	52.3	13.1
L-Arg- β -Nap	—	—	—
L-Asp- β -Nap	—	—	—
L-Gln- β -Nap	7.8	8.2	1.1
Gly- β -Nap	7.2	4.8	0.7
L-His- β -Nap	—	—	—
L-Leu- β -Nap	7.5	11.7	1.6
L-Lys- β -Nap	—	—	—
L-Met- β -Nap	—	—	—
L-Phe- β -Nap	—	—	—
L-Pro- β -Nap	1.1	39.7	36.1
L-Ser- β -Nap	—	—	—

L-alanine and L-proline effectively from L-alanine β -naphthylamide and L-proline β -naphthylamide respectively (Table 2). The K_m for DL-alanine β -naphthylamide is twice that of the L-isomer; this means that the D-isomer is neither a substrate nor a competitive inhibitor.

Effects of metal ions, chemical reagents and inhibitors

The enzyme was completely inhibited by Zn^{2+} , Cu^{2+} and Cd^{2+} ions. In contrast, it was activated by Mn^{2+} and Mg^{2+} . EDTA did not inhibit, but apparently activated the enzyme activity, whereas EGTA and *o*-phenanthroline weakly inhibited. Thiol-enzyme inhibitors (*N*-ethylmaleimide and *p*-chloromercuribenzoate) inhibited the enzyme strongly, but serine-proteinase inhibitors (phenylmethanesulphonyl fluoride and 1-chloro-4-phenyl-3-tosylamidobutan-2-one) were not inhibitory. The enzyme activity inactivated by *p*-chloromercuribenzoate was partially recovered by the addition of 2-mercaptoethanol, but that inactivated by *N*-ethylmaleimide was not recovered. The enzyme was not affected by various naturally occurring aminopeptidase inhibitors, i.e. amastatin, bestatin, pepstatin and leupeptin.

Optimal pH and optimal temperature

The optimal pH of the enzyme was pH 7.5–8.0 when L-alanine β -naphthylamide was used as substrate. Borate buffer might be inhibitory to the enzyme. The optimal temperature of the enzyme was about 35–40 °C (although *X. citri* cannot tolerate temperatures over 30 °C). The enzyme was stable towards heat shock (at least 55 °C for 10 min).

DISCUSSION

The alanyl-sulphamoyl bond is not common in Nature, and the enzyme that attacks such a bond was hitherto unknown. In the course of studies on the mechanism of action of the nucleoside antibiotic ascamycin (Osada & Isono, 1985) we have been able to discover the presence of an enzyme having such activity in *X. citri*.

The enzyme purified from *X. citri* was named Xc aminopeptidase. It liberates alanine and proline from their respective β -naphthylamides, but does not attack

the β -naphthylamides of various other amino acids tested. It is also specific to alanyl- and prolyl-peptides. The enzyme that releases an *N*-terminal proline from peptides is classified as proline iminopeptidase (EC 3.4.11.5). Therefore, Xc aminopeptidase is considered to belong to this class.

Xc aminopeptidase is a monomer of M_r 38000. This value is smaller than those of the reported proline iminopeptidases, i.e. the M_r values of proline iminopeptidases are 58000 (monomer) from *Bacillus megaterium* (Yoshimoto *et al.*, 1983), 220000 (tetramer) from apricot seed (Ninomiya *et al.*, 1982), 300000 (pentamer or hexamer) from bovine kidney (Khilji & Bailey, 1978) and 300000 (polymericity not described) from pig kidney (Nordwig & Mayer, 1973).

Sarid *et al.* (1959) first reported that proline iminopeptidase from *E. coli* was activated by Mn^{2+} . Xc aminopeptidase was also activated by Mn^{2+} , although the enzyme was completely inhibited by Zn^{2+} and Cd^{2+} . Yoshimoto *et al.* (1983) reported that proline iminopeptidase from *Bacillus megaterium* was markedly inhibited by *p*-chloromercuribenzoate, and that the inhibition was recovered by the addition of 2-mercaptoethanol. Xc aminopeptidase was also inhibited by *p*-chloromercuribenzoate and re-activated partially by 2-mercaptoethanol. Xc aminopeptidase was not inhibited by EDTA, phenylmethanesulphonyl fluoride, 1-chloro-4-phenyl-3-tosylamidobutan-2-one and naturally occurring aminopeptidase inhibitors such as amastatin, antipain, bestatin and leupeptin. Several properties of Xc aminopeptidase resemble those of iminopeptidases from *Bacillus* and other sources. However, the M_r of Xc aminopeptidase is different from that of the reported proline iminopeptidases. The unique property of Xc aminopeptidase is to liberate alanine from ascamycin and alanine β -naphthylamide effectively. Some proline iminopeptidases have been reported to hydrolyse the synthetic substrate L-proline β -naphthylamide (Nordwig & Mayer, 1973; Khilji & Bailey, 1978; Ninomiya *et al.*, 1982; Yoshimoto *et al.*, 1983). Therefore it may be worthwhile to test the reported proline iminopeptidases to see whether they hydrolyse ascamycin.

The enzymic detoxification of antibiotics has been reviewed by Davies & Smith (1978). Resistance to antibiotics is known to be achieved by enzymes such as aminoglycoside phosphotransferase (Dowding, 1977), aminoglycoside acetyltransferase (Brzezinska *et al.*, 1972), aminoglycoside adenyltransferase (Benveniste & Davies, 1971), chloramphenicol acetyltransferase (Shaw, 1974) and β -lactamase (Sykes & Matthew, 1976). In contrast, little is known about the enzymes that are able to increase the susceptibility to antibiotics. It is noteworthy that *X. citri* is susceptible to ascamycin because of the presence on the cell envelope of the enzyme Xc aminopeptidase that catalyses the dealanylation of ascamycin, which may be requisite for the membrane transport of the molecule.

Although the physiological function of the enzyme is not yet known, the finding that a plant-pathogenic bacterial *Xanthomonas* species has the unique hydrolysing enzyme on the cell surface and that dealanylation of ascamycin can stimulate uptake of the toxic moiety raised the possibility of selectively targeting toxic agents by alanylation or prolylation of the active compound against the organisms possessing the enzyme. In preliminary experiments (H. Osada & K. Isono, unpublished work),

employing the immunofluorescence technique, we have shown that simian-virus-40-transformed Balb3T3 cells have larger amount of immunologically related proteins on the cell surface compared with normal cells, and furthermore we have observed higher ascamycin-dealanylation activity in simian-virus-40-transformed cells compared with normal cells.

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