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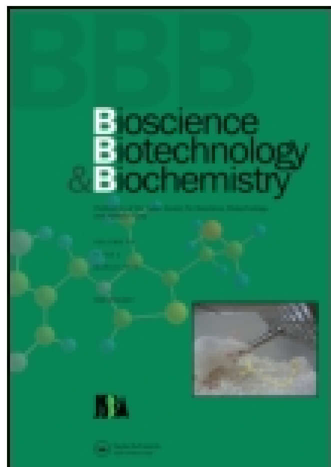

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Note

Hydrolysis of S-2-Aminoethylcysteinyl Peptide Bond by *Achromobacter* Protease I

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The substrate specificity of *Achromobacter* protease I (API) was examined for S-2-aminoethyl(AE)cysteinyl bonds in Bz-AEC-OMe/OEt, Bz-AEC-NH_2, and AE-insulin B chain. The protease hydrolyzed all of the tested AE-cysteinyl bonds at the same rate as that of lysyl bonds. Kinetic parameters were estimated for this hydrolysis reaction.

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AEC is isosteric to L-lysine. Trypsin is known to hydrolyze a peptide bond at the carboxyl side of AEC residues in addition to those of lysine and arginine residues,[1,2] though the rate of hydrolysis is much slower than for lysyl bonds.[1–4] *Achromobacter* protease I (EC 3.4.21.50, API) is a mammalian-type serine protease which specifically hydrolyzes peptide bonds at the carboxyl side of lysine residues,[5] and has been employed as a useful tool for the fragmentation of peptide chains in protein sequence analysis.[6] Kawata *et al.* have reported that the hydrolysis of AE-cysteinyl bonds in an S-2-aminoethylated peptide bonds was extremely useful to sequence RNase T_2.[7] To estimate the susceptibility of AE-cysteinyl bonds, we investigated the hydrolysis with API of the methyl ester, ethyl ester, and amide of AEC, and reduced and S-2-aminoethylated insulin B chain.

API was purified as described previously.[8] The ester and amide of Bz-AEC were synthesized from AEC hydrochloride *via* N^{ω}-Z-N^{α}-carboxyl-(S-2-aminoethyl)-cysteine anhydride as described by Wang and Carpenter.[9] Elemental analyses of the AEC derivatives synthesized are: Bz-AEC-OMe (syrup) *Anal.* Found: C 42.93, H 5.15, N 7.50. Calcd. for $C_{13}H_{18}N_2O_3S \cdot HBr$: C 42.97, H 5.27, N 7.72%; Bz-AEC-OEt (syrup), *Anal.* Found: C 44.40, H 5.58, N 7.30. Calcd. for $C_{14}H_{20}N_2O_3S \cdot HBr$: C 44.57, H 5.61, N 7.42%; Bz-AEC-NH_2 (crystallized from ethanol–ether, mp 183–185°C), *Anal.* Found: C 41.30, H 5.15, N 12.0. Calcd. for $C_{12}H_{17}N_3O_2S \cdot HBr$: C 41.40, H 5.17, N 12.06%.

S-2-Aminoethylated porcine insulin was prepared by reduction with 2-mercaptoethanol, followed by the reaction with ethyleneimine (Sogo Pharmaceutical Co., Ltd., Japan) by the method of Rall *et al.*[10] S-Alkylated insulin A and B chains were separated by reverse-phase HPLC using a linear gradient of 0–50% 2-propanol–acetonitrile (7 : 3, v/v) on a Cosmosil 5C18-AR column (1.0 × 25 cm, 5 μm) at a flow rate of 1.0 ml/min at ambient temperature. The purity of AE-insulin B chain thus obtained was confirmed by amino acid analysis.

Table summarizes kinetic parameters for the API-catalyzed hydrolysis of the ester and amide of Bz-AEC and their lysine counterparts. The k_{cat}/K_m (app) determined with Bz-AEC-OMe and Bz-AEC-OEt were about 2.5-fold higher than that of Bz-Lys-OMe. This is mainly due to an increase in k_{cat} (5-fold) since K_m was increased 2-fold. On the other hand, k_{cat}/K_m was very close for Bz-AEC-NH_2 and Bz-Lys-NH_2. These results indicate that AE-cysteinyl peptide bond can be hydrolyzed by the action of API at a rate similar to that of lysyl peptide bonds. To confirm the sensitivity of AE-cysteinyl bond to API, AE-insulin B chain (350 nmol) was digested with API (0.88 nmol) in 1.1 ml of 50 mM Tris–HCl buffer (pH 9.0) at 30°C. After 2, 4, and 8 h of incubation, samples (0.2 ml) were withdrawn, heated (98°C, 5 min), and analyzed by reverse-phase HPLC (Fig.). By the amino acid composition and the N-terminal amino acid[11] analysis, the peptide in each fraction (AP-1–AP-4) was identified as follows: AP-1, Phe[1]-AEC[7] (75.0%); AP-2, Gly[8]-AEC[19] (97.8%); AP-3, Gly[20]-Lys[29] (79.2%); P-4, Ala[30] (95.3%) (Peptide yields in 4 h of digestion are presented in parentheses and the N-terminus is underlined). The results suggests that peptide bond was cleaved at AEC[7]-Gly[8], AEC[19]-Gly[20], and Lys[29]-Ala[30] (Fig.). In 4 h digestion, no undegraded AE-insulin B chain remained, indicating that the rate of cleavage is nearly equal for the two AE-cysteinyl and a lysyl bond in S-2-aminoethylated insulin B chain. This is compatible with the result reported for the API-digestion of AE-hemolysin from *Vibrio parahaemolyticus* bearing 2 AEC and

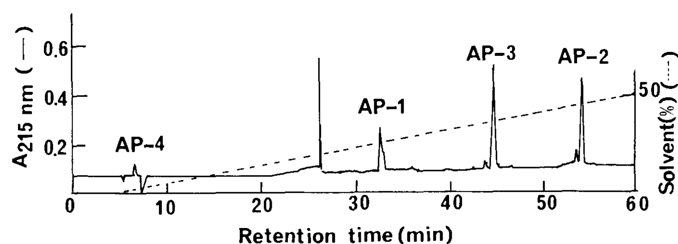
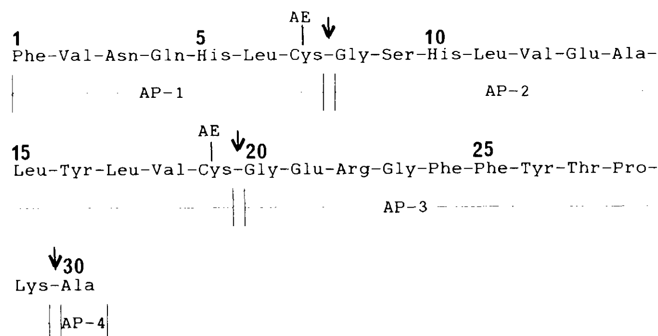


Fig. Separation of *Achromobacter* Protease I Digest of S-2-Aminoethylated Insulin B Chain.

The 4-h digest (34 nmol) obtained at pH 9.0 was chromatographed on a TSK-gel ODS-120T (0.46 × 25 cm) and eluted with a linear gradient of 0—50% 2-propanol–acetonitrile (7 : 3, v/v) in 0.05% TFA for 1 h at a flow rate of 0.5 ml per min. The respective fractions were identified by amino acid and N-terminal amino acid analyses. The results of these analyses are as follows.



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Abbreviations: AE, S-2-aminoethyl; AEC, S-2-aminoethyl-L-cysteine; Bz, benzoyl; Z, benzyloxycarbonyl; OMe, methyl ester; OEt, ethyl ester; TFA, trifluoroacetic acid.

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Table Kinetic Constants for Hydrolysis of Esters and Amides by *Achromobacter* Protease I and Bovine trypsin

The enzyme assay was done at 30°C and at a given pH according to the spectrophotometric method described in refs. 4, 9, and 13. A reaction mixture contained (a) 0.01–0.23 mM substrates and 2.77 nM enzyme in 3.0 ml of 80 mM Tris–HCl buffer, pH 8.25 (b) 0.05–1.0 mM substrates and 1.46 μM enzyme in 3.0 ml of 100 mM Tris–HCl buffer, pH 9.0 (c) 0.04–1.0 mM substrates and 76.4 nM enzyme in 3.0 ml of 80 mM Tris–HCl buffer, pH 8.25. Calculations were based on the molar absorptivity differences of 830 $cm^{-1} M^{-1}$ (253 nm) for Bz-AEC-OMe (or OEt),[9] 620 $cm^{-1} M^{-1}$ (253 nm) for Bz-AEC-NH_2,[9] 1160 $cm^{-1} M^{-1}$ (253 nm) for Bz-Lys-OMe,[13] and 750 $cm^{-1} M^{-1}$ (253 nm) for Bz-Lys-NH_2.[4] The kinetic parameters were obtained with a plot of 1/v vs. 1/s. For the calculation of kcat, the molecular weights of *Achromobacter* protease I and bovine trypsin were assumed to be 28,000 and 24,000, respectively.

Substrates	*Achromobacter* protease I				Bovine trypsin			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/k_m ($mM^{-1} s^{-1}$)	pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)	pH
Bz-AEC-OMe	0.1	1,200	12,000	8.25[a]	0.37	30.6	82.7	8.25[c]
Bz-AEC-OEt	0.1	1,400	14,000	8.25[a]	0.37	37.3	100.8	8.25[c]
Bz-Lys-OMe	0.05	264.0	5,280	8.25[a]	0.063	28.8	457.1	8.25[c]
Bz-AEC-NH_2	0.19	1.08	5.68	9.0[b]	4.6[d]	0.32[d]	0.07[d]	9.0[d]
Bz-Lys-NH_2	0.32	1.54	4.81	9.0[b]	4.7[d]	1.7[d]	0.36[d]	9.0[d]

[d] From Wang and Carpenter.[4]

10 lysine residues.[12]

API is in sharp contrast to bovine trypsin, which has a very low activity on the AEC-X bond. Presumably, the limited specificity of API for lysine permits the retention of a high peptidase activity to the peptide bond that AEC isosteric to lysine is involved in.

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