STRUCTURE DETERMINATION OF ALPHOSTATIN, A NOVEL ALKALINE PHOSPHATASE INHIBITOR

Sir:

Alphostatin, a novel bovine liver alkaline phosphatase inhibitor, was isolated from microbial fermentation broth and its physico-chemical properties and biological activities were reported in the preceding paper[1]. This paper deals with the structure determination of alphostatin. Alphostatin was obtained as colorless powder. MP 203~205°C (dec), *Anal* Calcd for $C_{25}H_{45}N_6O_{13}P \cdot H_2O$: C 43.73, H 6.90, N 12.24. Found: C 43.46, H 6.91, N 12.17. It gave positive Rydon-Smith reaction and weakly positive ninhydrin and acid molybdate, but negative 2,4-dinitrophenylhydrazine, Pauly, Sakaguchi and Ehrlich reactions. The UV spectrum of alphostatin showed only end absorption. Absorptions in the IR spectrum of alphostatin were seen at ν_{max} (KBr) cm^{-1} 2800~2400, 1720, 1670, 1640, 1555, 1250 and 1200. On paper electrophoresis (600V, 1 hour) using formic acid - acetic acid - water (25:75:900), alphostatin moved toward the cathode; Rm 0.058 (Ala=1). These data suggest that alphostatin is an acidic peptide with a free amino-terminal. ^1H NMR spectrum of alphostatin in CF_3COOD is as follows: δ 4.2~5.3 (7H), 2.6~3.1 (4H), 1.9~2.6 (6H), 1.3~1.9 (4H), 0.6~1.3 (12H) and ^{13}C NMR spectrum in the same solvent is as follows: δ 182.31 (s), 181.55 (s), 178.00 (s), 175.44 (s), 174.96 (s), 172.59 (s), 171.16 (s), 68.53 (t), 61.62 (d×2), 55.92 (d×2), 54.46 (d), 39.29 (d×2), 32.49 (t), 31.70 (t), 29.45 (t), 27.88 (t), 26.71 (t), 26.24 (t), 16.02 (q), 15.50 (q), 11.67 (q), 11.24 (q).

Acid hydrolysis of alphostatin with 6 M HCl at 110°C for 24 hours gave four ninhydrin positive compounds; isoleucine, glutamic acid, serine and a small amount of unknown compound. The structure of the unknown compound was elucidated to be isoleucyl-isoleucine with the NMR spectroscopy and the mass spectroscopy (M^+, *m/z* 244): NMR (D_2O, TMS) δ 4.64 (1H, d), 4.28 (1H, d), 2.1~2.65 (2H), 1.5~2.3 (4H), 1.1~1.7 (12H). This fact suggests that the inhibitor contains the partial structure of Ile-Ile in its structure. The molar ratio of serine, glutamic acid and isoleucine was determined to be 1:2:2 by the amino acid analysis of acid hydrolysate. In addition to these amino acids and dipeptide, the acid hydrolysate of alphostatin also contained inorganic phosphoric acid which was detected with ammonium moribdate - ferrous sulfate. To determine the mole number of phosphoric acid contained in an alphostatin molecule, we tried to hydrolyze the latter by alkaline phosphatase originated from chick intestine. Because of the insensitivity of this enzyme to alphostatin, this enabled us to analyze its phosphorous quantity with ammonium moribdate - ferrous sulfate. The hydrolysis of alphostatin with chick intestine alkaline phosphatase in 0.1 M carbonate buffer (pH 9.0) contained 0.2 M $MgCl_2$ at 37°C for 22 hours gave phosphoric acid by 3.8~4.2% (w/w) of alphostatin (calcd 4.6%) and dephospho-alphostatin (I), which suggested that 1 mol of phosphoric acid was contained in its molecule and formed phosphoric acid ester with serine. The amino acid sequence was elucidated

Fig. 1. Chemical degradation of dephospho-alphostatin.

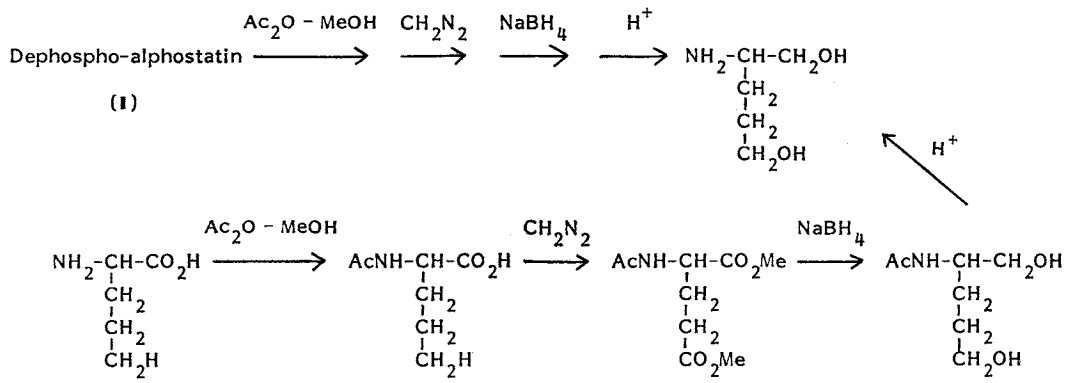
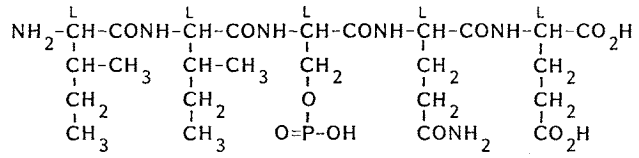


Fig. 2. Structure of alphostatin.



by the use of **I**. When **I** was digested with leucine aminopeptidase at 37°C, isoleucine was formed immidiately and 24 hours after incubation, isoleucine, serine, glutamine and glutamic acid were formed. The result of enzymatic hydrolysis of **I** enabled us to deduce that *N*-terminal amino acid was isoleucine and that its next was also isoleucine because Ile-Ile was obtained by acid hydrolysis. Since **I** was hydrolyzed by leucine aminopeptidase completely, it was deduced that the configurations of these amino acids were all L-form. The hydrolysis with carboxypeptidase A at 37°C for 2 hours gave glutamine and glutamic acid at the same time. Then in order to determine the *C*-terminal amino acid, **I** was converted to an alcohol derivative. After the acid hydrolysis, the formed aminoalcohol was compared with the authentic sample obtained from glutamic acid and glutamine by the same treatment. The aminoalcohol obtained from **I** was identical with the one from glutamic acid, but not with one from glutamine (Fig. 1).

The results obtained from these enzymatic hydrolysis and the chemical degradations elucidated the structure of the inhibitor as Fig. 2. Although DOELLGAST and FISHMAN reported several L-leucine containing peptide inhibitors without phosphoserine, their potencies were very low[2]. Phosphoserine may be essential part for the potent inhibitory activity of alphostatin.

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2) DOELLGAST, G. J. & W. H. FISHMAN: Inhibition of human placental-type alkaline phosphatase variants by peptides containing L-leucine. Clin. Chim. Acta 75: 449~454, 1977