

very likely in view of their mutagenicity that these compounds, especially the carbamate type compounds, have a carcinogenic effect.

**Acknowledgement** The authors are grateful to Professor M. Sekiya, Shizuoka College of Pharmacy, for valuable advice and for preparing the N-nitroso compounds tested.

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[*Chem. Pharm. Bull.*  
**29**(6)1762—1764(1981)]

### Stereoisomeric Alanine Peptides as Substrates for Human Spleen Fibrinolytic Proteinase (SFP)<sup>1)</sup>

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(Received December 20, 1980)

Four kinds of stereoisomeric Z-Ala-Ala-OMe and eight kinds of stereoisomeric Z-Ala-Ala-Ala-OMe were tested as substrates for human spleen fibrinolytic proteinase (SFP) in comparison with porcine pancreatic elastase. Both enzymes exhibited esterase activity towards not only Z-L-Ala-L-Ala-L-Ala-OMe (VI) but also Z-D-Ala-L-Ala-L-Ala-OMe (VII). The rate of esterolysis of VI by elastase was only about twice the rate of esterolysis by SFP although the rate of amidolysis of Suc-L-Ala-L-Ala-L-Ala-pNA (XVI) by elastase was tenfold faster than that by SFP.

**Keywords**—synthetic substrate; stereoisomeric alanine peptide; human spleen fibrinolytic proteinase; porcine pancreatic elastase; rate of hydrolysis

Nagamatsu has described the isolation and purification of a neutral proteinase capable of degrading fibrin and fibrinogen from human spleen tissue (SFP).<sup>2)</sup> Later, Okamoto *et al.*<sup>3)</sup> reported that the properties of this enzyme, as far as examined, were similar to those of elastase, especially human leucocyte elastase, except for the substrate specificity. For instance, the activity of SFP to release *p*-nitroaniline from the substrate, Suc-L-Ala-L-Ala-L-Ala-pNA, is much poorer than that of porcine pancreatic elastase. Further studies on SFP are under way in our laboratories. In the present paper, we report on the properties of stereoisomeric alanine peptides as substrates for SFP and porcine pancreatic elastase, and compare the esterolytic and amidolytic activities of SFP and porcine pancreatic elastase.

### Experimental

**Materials**—The synthesis of stereoisomeric Z-Ala-Ala-OMe and Z-Ala-Ala-Ala-OMe was reported previously.<sup>4,5)</sup> Suc-L-Ala-L-Ala-L-Ala-pNA, HCl·L-Ala-L-Ala-L-Ala-pNA, L-Ala-pNA and Bz-L-Ala-OMe were purchased from the Protein Research Foundation (Osaka).

**Enzyme Preparation and Assay Procedure**—SFP was purified from human spleen by ultracentrifugation, chloroform treatment, salting out with 50% saturated ammonium sulfate and repeated gel-filtration on Sephadex G-100. The resulting preparation possessed fibrinolytic activity, and showed a single band on polyacrylamide gel electrophoresis at pH 4.5.<sup>2,3)</sup> Pancreatic elastase (porcine type III) was purchased from Sigma Chem. Co. (St. Louis) and dissolved in Tris-HCl buffer (0.1 M, pH 8.0) containing 2 M NaClO<sub>4</sub> in the same manner as SFP.

Amidolytic activity was assayed by measuring the pNA (E<sub>410</sub>) released in 10 min according to the method described by Erlanger *et al.*<sup>6)</sup>

Esterolytic activity was assayed by measuring methanol released in 10 min according to the method developed by Siegelman *et al.*<sup>7)</sup> and modified by Johnson *et al.*<sup>8)</sup>

All substrates were dissolved in Tris-HCl buffer (0.1 M, pH 8.0), and enzymatic hydrolysis was carried out at 37°. Enzyme solution added was one-tenth of the volume of reaction mixture.

### Results and Discussion

Results on the rates of esterolysis of stereoisomeric alanine peptide esters by SFP and porcine pancreatic elastase are summarized in Table I. Esterase activity of both enzymes towards Bz-L-Ala-OMe (I) was negligible. In the series of Z-Ala-Ala-OMe, small amounts of methanol were released from Z-L-Ala-L-Ala-OMe (II) and Z-D-Ala-L-Ala-OMe (III) by

TABLE I. Comparative Esterolysis by SFP and Porcine Pancreatic Elastase

Substrate	Released methanol (mm)/mg enzyme/10 min	
	SFP	Elastase
Bz-L-Ala-OMe (I)	<0.04	<0.04
Z-L-Ala-L-Ala-OMe (II)	0.07	0.35
Z-D-Ala-L-Ala-OMe (III)	0.04	0.12
Z-L-Ala-D-Ala-OMe (IV)	0	0
Z-D-Ala-D-Ala-OMe (V)	0	0
Z-L-Ala-L-Ala-L-Ala-OMe (VI)	0.71	2.4
Z-D-Ala-L-Ala-L-Ala-OMe (VII)	0.48	2.16
Z-D-Ala-D-Ala-L-Ala-OMe (VIII)	<0.04	<0.04
Z-L-Ala-D-Ala-L-Ala-OMe (IX)	<0.04	<0.04
Z-L-Ala-L-Ala-D-Ala-OMe (X)	0	0
Z-D-Ala-L-Ala-D-Ala-OMe (XI)	0	0
Z-L-Ala-D-Ala-D-Ala-OMe (XII)	0	0
Z-D-Ala-D-Ala-D-Ala-OMe (XIII)	0	0

The final concentration of esters was  $3 \times 10^{-3}$  M.

The figures in the table were calculated on the assumption that all protein in the purified preparation was SFP.

the action of both enzymes. Esterase activity of porcine pancreatic elastase towards the dipeptides, (II) and (III), was much higher than that of SFP. The conformation of the dipeptide, (II) and (III), is not so suitable as substrates for SFP as well as porcine pancreatic elastase. The isomers containing D-alanine at the C-terminus, L-D (IV) and D-D (V), were not hydrolyzed at all by either enzyme to release methanol. In the series of stereoisomeric Z-Ala-Ala-Ala-OMe, the ester of the L-L-L form (VI) was hydrolyzed by both enzymes with the highest rate among the compounds tested. This results is compatible with the previous report that Ac-L-Ala-L-Ala-L-Ala-OMe is a good substrate for elastase.<sup>9)</sup> The conformation of tripeptide (VI) might be more favorable for reacting with the binding site and catalytic site of both enzymes than that of dipeptides described above. The isomer, D-L-L (VII) was

also hydrolyzed by both enzymes. The rate of esterolysis of VI by both enzymes was slightly faster than that of VII. The presence of a D-alanine residue in VII had more effect on SFP than elastase as regards decreasing the rate of hydrolysis. No significant esterase activity of either enzyme towards the isomers, D-D-L (VIII) and L-D-L (IX), was observed, although the amino acid residue at the C-terminus of those substrates was L-form. This might be explained by assuming that the approach of those substrates to the active centers of the enzymes was prevented by the side chains of D-alanine residues of the substrates, or that D-alanine residues of VIII and IX inhibited the active centers of the enzymes. With regard to the substrates containing D-alanine at the C-terminus, esters of the isomers, L-L-D (X), D-L-D (XI), L-D-D (XII) and D-D-D (XIII) were not hydrolyzed by either enzyme, as expected. However, whether these substrates can react with the binding sites of the enzymes is not known yet. The results show that the rate of hydrolysis of stereoisomeric substrates by SFP exhibited a pattern similar to that of porcine pancreatic elastase.

The next series of studies was performed in order to clarify the relative potencies of esterolytic activity and amidolytic activity of SFP and porcine pancreatic elastase. The rates of amidolysis of *p*-nitroanilide substrates by SFP and elastase are presented in Table II. Amido-

TABLE II. Comparative Rates of Amidolysis by SFP and Porcine Pancreatic Elastase

Substrate	Release <i>p</i> NA ( $\mu$ M)/mg Enzyme/10 min	
	SFP	Elastase
L-Ala- <i>p</i> NA (XIV)	0	0
L-Ala-L-Ala-L-Ala- <i>p</i> NA (XV)	<1	<1
Suc-L-Ala-L-Ala-L-Ala- <i>p</i> NA (XVI)	12	175

The final concentration of substrates was  $1.5 \times 10^{-3}$  M.

lytic activity of SFP towards alanine peptide *p*-nitroanilides paralleled that of elastase, although the activity of SFP was much lower than that of elastase. The results in Table I and II show that the esterase activity of elastase towards the substrate (VI) was only twice that of SFP (4800 and 2150  $\mu$ M released methanol/ $\mu$ M enzyme/min respectively), whereas the amidolytic activity of elastase towards XVI was about tenfold higher than that of SFP (350 and 36  $\mu$ M released *p*-nitroaniline/ $\mu$ M enzyme/min, respectively). It was concluded that the ester (VI) was not a highly specific substrate for elastase (although Gertler and Hofmann<sup>9</sup>) reported that Ac-L-Ala-L-Ala-L-Ala-OMe was a highly specific esteratic substrate for elastase), but the *p*-nitroanilide (XVI) was fairly specific for elastase.

#### References and Notes

- 1) Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1976). Z=benzyloxycarbonyl, Bz=benzoyl, OMe=methyl ester, Suc=succinyl, *p*NA=*p* nitroanilide, Ac=acetyl
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