PEPTIDE ALDEHYDES INHIBITING CHYMOTRYPSIN

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SUMMARY Peptide aldehydes, in which the argininal moiety of leupeptin Ac (Ac-Leu-Leu-argininal) was replaced by phenylalaninal, tyrosinal or tryptophanal, were synthesized. These compounds exhibited potent inhibition on the proteolytic activity of chymotrypsin in contrast with leupeptin which was reported to inhibit trypsin. These results demonstrated a marked relationship between the C-terminal amino aldehyde moiety of these inhibitors and the substrate specificities of chymotrypsin and trypsin. The importance of the aldehyde group for activity was reinvestigated.

Recently, Umezawa et al. isolated leupeptins Pr-LL and Ac-LL, which competitively inhibited the proteolytic activity of trypsin, from the culture of various strains of Actinomycetes. Their chemical structures were elucidated to be propionyl- and acetyl-L-leucyl-L-leucyl-DL-argininal respectively (1, 2, 3). Investigations on the relationship between the structure and the activity of these compounds, using various synthetic analogs, showed that the aldehyde group and guanidyl group of the argininal moiety are essential for this inhibitory effect (1, 4). Supposedly, the guanidyl group is related to the specific inhibition of trypsin in connection with specificity of this proteinase, which cleaves the peptide linkage involving the carboxyl groups of lysine and arginine. In other words, the guanidyl group in leupeptins may have an affinity for the specific site of trypsin in competition with its substrate. Supporting this, leupeptin did not inhibit the action of chymotrypsin, another representative proteolytic enzyme.

It is well known that chymotrypsin is similar to trypsin in its properties as a protein and in the supposed proteolysis mechanism (5, 6). However, its substrate specificity is entirely different from that of trypsin. Chymotrypsin cleaves peptide bonds mainly at the carboxyl side of aromatic amino acids (phenylalanine, tyrosine, tryptophan). The failure of leupeptins to act on chymotrypsin may be due to the difference in this enzyme specificity. To confirm this assumption, we synthesized the leupeptin Ac analogs in which the argininal residue was replaced by phenylalaninal, tyrosinal or tryptophanal, and examined their inhibitory effect on chymotrypsin. All of the synthesized analogs showed marked inhibition of chymotrypsin as expected.

MATERIALS AND METHODS

α-Chymotrypsin (thrice-crystallized, salt free) and trypsin (twice-crystallized, salt free) were purchased from Sigma Chemical Co. Ac-L-Tyr-OEt was obtained from the Peptide Center of the Institute for Protein Research. Casein was purified according to the procedure described by Norman (7). TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) was prepared according to the method of Schoellmann and Shaw (8).

Five acetyl peptide aldehydes were synthesized in essentially the same way already reported (9). The N-carbobenzoxy aromatic amino acid was converted to acid imidazolide with carbodiimidazole in anhydrous tetrahydrofuran. The imidazolide was reduced to the corresponding aldehyde without isolation from the reaction mixture by lithium aluminium hydride at -20°C. The resulting aldehyde was converted to its semicarbazone to protect the aldehyde group during the subsequent synthetic processes. The carbobenzoxy group was removed by 25% hydrogen bromide in acetic

acid, except in the case of tryptophan. Here hydrogenolysis was applied to avoid oxidative destruction by the strong acid. The crude, unblocked product was then coupled with carbobenzoxy-L-leucine hydroxysuccinimide ester. After decarbobenzoxylation by hydrogenolysis, carbobenzoxy-L-leucine hydroxysuccinimide ester was again coupled. The carbobenzoxy-L-leucyl or carbobenzoxy-L-leucyl-L-leucyl aromatic amino acid aldehyde semicarbazone thus obtained were hydrogenated to remove the carbobenzoxy group and acetylated by acetic anhydride and triethylamine. Finally the acetyl peptide aldehyde semicarbazone was treated with 2.3% formaldehyde in 0.15 N methanolic hydrochloric acid at room temperature for 12 hours to reproduce the aldehyde group.

Acetyl-L-leucyl-L-leucyl-phenethylamine was synthesized by stepwise elongation from phenethylamine. 3-(Acetyl-L-leucyl-L-leucyl)-amido-4-phenyl-2-butanone was also synthesized by stepwize elongation from 3-amino-4-phenyl-2-butanone ethylene ketal, followed by deblocking the ketal group with 90% trifluoroacetic acid. 3-amino-4-phenyl-2-butanone ethylene ketal was obtained as follows: TPCK was dechlorinated by hydrogenolysis to give the corresponding methyl ketone. After the carbonyl group was blocked by forming ethylene ketal, the tosyl group was removed by sodium in liquid ammonia. The purity of the synthesized materials was checked by elementary analysis and thin layer chromatography.

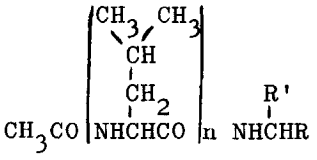
The proteolytic activity was determined as follows: One ml of 2.0% casein solution, 0.5 ml of 0.01 M borate buffer of pH 7.4 and 0.3 ml of ethanol, with and without inhibitor, were mixed. After 3 minutes preincubation, 0.2 ml chymotrypsin solution, (40 μg/ml, containing 0.05 ml of 0.02 M $CaCl_2$) was added and the incubation was continued for 30 minutes at 37°C. The reaction was stopped by the addition of 2.0 ml of 1.7 M perchloric acid.

After 1 hour, the precipitates were discarded by centrifugation and the extinction of the supernatant was read at 280 mμ. The rate of hydrolysis of Ac-L-Tyr-OEt by chymotrypsin was determined following the method of Schwert and Takenaka (10).

RESULTS AND DISCUSSION

Table 1 shows that all of these synthesized acetyl tripeptide derivatives, Ac-Leu-Leu-tryptophanal (V), Ac-Leu-Leu-tyrosinal (IV) and Ac-Leu-Leu-phenylalaninal (II), inhibited chymotrypsin at comparable levels of concentration as leupeptin had inhibited trypsin (1). However trypsin was not inhibited, showing that their specificity with respect to the two proteolytic enzymes is just the opposite of leupeptin's. The inhibition increased in the order : Ac-Leu-Leu-phenylalaninal (II) $<$ Ac-Leu-Leu-tyrosinal (IV) $<$ Ac-Leu-Leu-tryptophanal (V). It is quite interesting that this order is parallel to that of the susceptibilities of aromatic amino acid esters to chymotrypsin : Phe $<$ Tyr $<$ Trp (11). It was also found that Ac-Leu-Leu-phenylalaninal (II) or Ac-Leu-Leu-tyrosinal (IV) inhibited the caseinolytic activity of chymotrypsin more strongly than Ac-Leu-phenylalaninal (I) or Ac-Leu-tyrosinal (III). A progressive increase in the number of leucyl residues of the synthesized compounds attends the strengthening of the inhibitory effect.

Umezawa *et al.* reported that an important role is played by the aldehyde group of the leupeptins. In order to confirm the similar essentiality of aldehyde group of Ac-Leu-Leu-phenylalaninal (II), we also synthesized Ac-Leu-Leu-phenethylamine (VI) and Ac-Leu-Leu-amidophenylbutanone (VII). Those two compounds had no effect on caseinolysis by chymotrypsin.



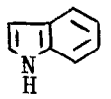
		n	R	R'	ID_{50} (μg/ml)
I	Ac-Leu-phenylalaninal	1	$-CH_2$ 	-CHO	> 300
II	Ac-Leu-Leu-phenylalaninal	2	$-CH_2$ 	-CHO	68
III	Ac-Leu-tyrosinal	1	$-CH_2$  OH	-CHO	300
IV	Ac-Leu-Leu-tyrosinal	2	$-CH_2$  OH	-CHO	33
V	Ac-Leu-Leu-tryptophanal	2	$-CH_2$ 	-CHO	17.2
VI	Ac-Leu-Leu-phenethylamine	2	$-CH_2$ 	-H	> 600
VII	Ac-Leu-Leu-amidophenylbutanone	2	$-CH_2$ 	$-COCH_3$	> 600

Table 1. Concentrations of the synthetic analogs of leupeptin Ac for 50% inhibition of caseinolysis by chymotrypsin

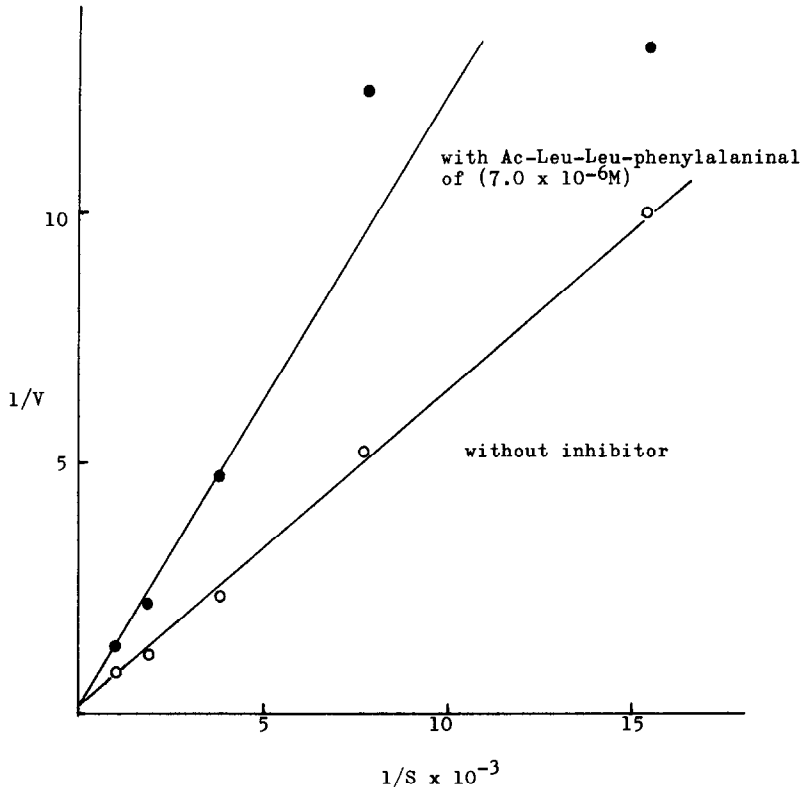


Fig. 1. Kinetics of inhibition by Ac-Leu-Leu-phenylalaninal chymotrypsin-Ac-L-Tyr-OEt system.

Kinetic studies have been carried out on the effect of Ac-Leu-Leu-phenylalaninal (II) on esterolysis Ac-L-Tyr-OEt by chymotrypsin. The typical results are shown in Fig. 1, using the Lineweaver-Burk plot. The inhibition is of the competitive type. The K_i of Ac-Leu-Leu-phenylalaninal (II) for esterolysis of Ac-L-Tyr-OEt by chymotrypsin was 5.2×10^{-5}M. On the other hand, the K_i value of leupeptin Ac on the hydrolysis of tosyl-L-Arg-OMe by trypsin was 8.1×10^{-5}M (1).

The application of this type of approach to other enzymes is being investigated.

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REFERENCES

1. Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishizuka, M., Takeuchi, T., and Umezawa, H.: J. Antibiotics 22: 558, 1969.
2. Kondo, S., Kawamura, K., Iwanaga, J., Hamada, M., Aoyagi, T., Maeda, K., Takeuchi, T., and Umezawa, H.: Chem. Pharm. Bull. 17: 1896, 1969.
3. Kawamura, K., Kondo, S., Maeda, K., and Umezawa H.: Chem. Pharm. Bull. 17: 1902, 1969.
4. Maeda, K., Kawamura, K., Kondo, S., Aoyagi, T., Takeuchi, T., and Umezawa, H.: J. Antibiotics 24: 402, 1971.
5. Steitz, T.A., Henderson, R., and Blow, D.M.: J. Mol. Biol. 46: 337, 1969.
6. Kasserra, H.P. and Laidler, K.J.: Can. J. Chem. 47: 4031, 1969.
7. Norman, P.S.: J. Exptl. Med. 106: 423, 1957.
8. Schoellmann, G., and Shaw, E.: Biochemistry 2: 252, 1963.
9. Shimizu, B., Saito, A., Ito, A., Tokawa, K., Maeda, K., and Umezawa, H.: J. Antibiotics in press.
10. Schwert, G.W. and Takenaka, Y.: Biochim. Biophys. Acta 16: 570, 1950.
11. Neurath, H., and Schwert, G.W.: Chem. Rev. 46: 69, 1950.