ACYL INTERMEDIATES IN PEPSIN AND PENICILLOPEPSIN CATALYZED REACTIONS.*

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Received December 27,1973

SUMMARY

Porcine pepsin and penicillopepsin catalyze transpeptidation reactions which involve the transfer in high yield of N-terminal leucine from Leu-Tyr-Leu, and Leu-Tyr amide to intact substrate with the subsequent release of Leu-Leu. With the latter substrate porcine pepsin also forms Leu-Leu-Leu. With penicillopepsin small amounts of Leu-Leu-Tyr-Leu and Leu-Leu-Tyr amide resp. were obtained as intermediates from Leu-Tyr-Leu and Leu-Tyr amide. No radioactivity was incorporated into Leu-Leu when Leu-Tyr-Leu and [^{14}C]-leucine (1:6) were incubated with the enzymes. It was concluded that the transpeptidation proceeds via covalent acyl intermediate.

Since the observation by Neumann et al (1) that pepsin can catalyze transpeptidation reactions which involve the transfer of a C-terminal amino acid and since evidence was presented (2) which strongly suggests that the reaction proceeds via a covalent amino intermediate most of the mechanisms of pepsin action proposed have included this intermediate as a central feature (see review by Clement, ref. 3). However, Sharon et al (4) showed that pepsin can catalyze ^{18}O exchange between a virtual substrate and water and concluded that this provided evidence for an acyl-intermediate. No support other than ^{18}O exchange has been forthcoming for this intermediate but some of the proposed mechanisms include an acyl intermediate while others account for the ^{18}O exchange without an acyl intermediate (3).

In this report we provide evidence for a new type of transpeptidation

* Supported by the Medical Research Council of Canada, Grant No. MT-1982.

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which involves the transfer of an N-terminal residue and appears to proceed via an acyl intermediate. The reaction is catalyzed by porcine pepsin and by penicillopepsin. The latter has been shown to be homologous to the porcine enzyme (5) and is similar in enzymatic properties (6). The present report shows that the two enzymes are also similar in their mechanism of action.

MATERIALS AND METHODS

Porcine pepsin (3 x cryst.) was obtained from Calbiochem. (Lots 46220 and 300001). Penicillopepsin was prepared from Penicillium janthinellum essentially according to the method of Sodek and Hofmann (7). Leu-Tyr amide was from Cyclo Chemical Co.; Leu-Tyr-Leu was from Mann Research Laboratories. It was purified before use by high-voltage electrophoresis at pH 3.6, 30V/cm for 90 min. [^{14}C]-leucine (10 mCi/mmole) was from Amersham-Searle Co. All amino acids and peptides were the L-isomers.

The reaction products from Leu-Tyr-Leu after enzymic digestion were separated by high-voltage electrophoresis in pyridine-acetate-formate-water, 7:16:16:960 (by volume) at pH 3.1, 30V/cm for 90 min, followed by chromatography in butanol:butylacetate:acetic acid:water, 130:6:30:50 (by volume). The products were eluted and identified by amino acid analysis and dansyl-Edman degradation (8).

The reaction products from Leu-Tyr amide were analyzed by a modification of a Beckman-Spinco Model 120C amino acid analyzer. Leucine and Leu-Leu were separated on a column of UR30 resin (20 x 0.9 cm) at 56° using 0.2 N sodium citrate at pH 4.3 and 5.25. The flow rate was 70 ml/hr and the buffer change occurred at 40 min. Leucine eluted at 29 min, Leu-Leu at 74 min. Tyr-amide was separated on a column of PA35 resin (3 x 0.9 cm) at 66°, with a 0.35 N sodium citrate buffer pH 5.25 and eluted at 80 min. The products were also separated by high voltage electrophoresis at pH 3.6 (9) for the identification of Leu-Leu-Tyr amide. Leu-Leu-Leu and Leu-Leu in the porcine pepsin experiments were separated on a UR30 resin column (60 x 0.9 cm) with 0.2 N sodium citrate buffers (pH 4.3 and 5.25) at 66° and a buffer change at 50 min.

Leu-Leu eluted at 104 min and Leu-Leu-Leu at 110 min.

RESULTS

Table I shows the effect of penicillopepsin on Leu-Tyr-Leu after prolonged incubation. The main product is Tyr-Leu, the hydrolysis product expected for cleavage of the N-terminal leucine. However, the amount of free leucine is only one-third of the Tyr-Leu and the second major product is Leu-Leu which must have arisen from a transpeptidation reaction.[1] Leu-Tyr is present in much smaller amounts than Tyr-Leu. Two peptides were found in small amounts only, Leu-Leu-Tyr-Leu and Tyr-Tyr. Both must be transpeptidation products. Fig. 1 shows a sketch of a high-voltage electropherogram of the incubation mixture of porcine pepsin with Leu-Tyr-Leu in comparison with that of penicillopepsin. Qualitatively and semi-quantitatively (as judged by eye) the reaction products are the same. Fig. 1 also shows the clear separation of most of the reaction products; complete separation was obtained by chromatography following electrophoresis. Table II shows the result of experiments in which the effect of both enzymes on Leu-Tyr-Leu in the presence of a 6-fold molar amount of [^{14}C]-leucine was studied. Only leucine and Leu-Leu were isolated in this case. The radioactivity in the dipeptide was barely above background (2-6 c.p.m. were counted) and the specific activity shows that less than 2% of the free leucine could have been incorporated in the dipeptide. The recovery of Leu-Leu as a percentage of the substrate used was comparable to that obtained in Table I. In a separate experiment it had been shown that free leucine has no significant effect on the reaction.

Fig. 2 shows a progress curve of the action of penicillopepsin on Leu-Tyr-amide. As with Leu-Tyr-Leu more Leu-Leu than free leucine was formed. The presence of a probable intermediate Leu-Leu-Tyr NH_2 was shown in a

[1] The results of this table are only semi-quantitative because the products were eluted from paper. However, the fact that the total recovery of leucine and tyrosine is about the same indicates that the amounts of each product recovered is representative of the amount present in the incubation mixture.

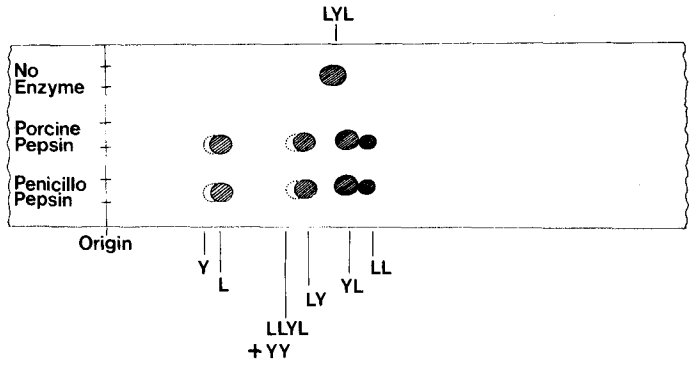


Figure 1. Electropherogram of products of pepsin action on Leu-Tyr-Leu.

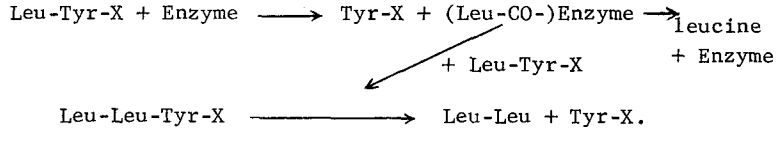
Conditions for penicillopepsin were as in Table I. Incubation with porcine pepsin was at pH 4.7 with 1 mg enzyme/ml.

separate experiment, since it could not be separated from the substrate on the amino acid analyzer. The curves of Tyr-amide, Leu-Leu and leucine show slight sigmoidicity up to about 50% of the reaction. This is due to activation by Leu-Leu (Wang, Dorrington and Hofmann, to be published).

Fig. 3 shows a similar experiment with porcine pepsin. Again Leu-Leu is formed, but in this case in smaller amounts than free leucine. No Leu-Leu-Leu-Tyr-amide could be detected, but an additional transpeptidation product Leu-Leu-Leu was separated on the amino acid analyzer. Together the transpeptidation products account for more than half the leucine cleaved from the substrate.

DISCUSSION

The experiments presented here give clear evidence that acyl intermediates are involved in at least some pepsin and penicillopepsin-catalyzed reactions. This evidence is based on the transpeptidation reactions involving transfer of an N-terminal leucine residue (acyl-transfer) according to this scheme:



(where X = -Leu or $-NH_2$)

TABLE I

Effect of penicillopepsin on Leu-Tyr-Leu

Products[b] identified	Recovery[c] (nanomoles)
Tyr-Leu	722
Leu-Leu	537
leucine	247
tyrosine	160
Leu-Tyr	194
Leu-Leu-Tyr-Leu	23
Tyr-Tyr	22
Leu-Tyr-Leu	not detected

a) Conditions: Leu-Tyr-Leu (3.2 μmoles) and penicillopepsin (0.2 mg) in pyridine-acetate-water (10:100:890, by volume) at pH 3.6 were incubated for 24 h at 35°.

b) → indicates that the peptide was degraded by the Edman method. N-terminals were identified as dansyl derivatives, C-terminals by amino acid analysis without hydrolysis after the appropriate number of degradations.

c) Recovery was calculated from the amino acid composition. No corrections were made for mechanical losses (guide strips, etc.) and destructive losses. The overall recovery of leucine and of tyrosine was 36%.

Although the experiments with Leu-Tyr-Leu do not exclude the possibility of a C-terminal transfer similar to the transpeptidations observed by Neumann et al. (1) acyl transfer is more likely for two reasons:

a) between 3 and 4 times as much Tyr-Leu as Leu-Tyr is formed; and b) Leu-Leu-Tyr-Leu has been clearly demonstrated. Also the action on Leu-Tyr amide is strictly analogous and here Leu-Leu can only arise from an acyl-transfer. Free leucine is not exchanged for the enzyme bound leucine, even when present in several fold excess. It is therefore reasonable to conclude that the

TABLE II

Effect of penicillopepsin and porcine pepsin on Leu-Tyr-Leu in presence of [^{14}C]-leucine.[a]

Expt.	Enzyme	Product[b]	nanomoles[c]	c.p.m.[c]	Specific activity (c.p.m./nmole)
1	penicillopepsin	leucine	3560	32200	9.0
		Leu-Leu	132	22	0.16
2	porcine pepsin	leucine	4770	45800	9.6
		Leu-Leu	134	12	0.09
3	no enzyme	leucine	2580	26600	10.2
		Leu-Leu	not found	-	-

a) Leu-Tyr-Leu (1.2 µmoles) and [^{14}C]-leucine (7.6 µmoles) were incubated in 0.5 ml pyridine-acetate with penicillopepsin (0.3 mg) at pH 3.6 or porcine pepsin (0.5 mg) at pH 4.7 for 24 h at 35°.

b) The products were eluted from paper after separation by electrophoresis and chromatography, and dissolved in 100 µl water. Samples were taken for amino acid analysis and for counting.

c) per 100 µl.

transfer proceeds via a covalent acyl intermediate. These experiments therefore provide convincing evidence for the involvement of an acyl intermediate in pepsin-catalyzed reactions. The transpeptidation proceeds in high yield. With penicillopepsin and Leu-Tyr amide as substrate about 80% of the leucine is present as Leu-Leu or Leu-Leu-Tyr amide when some 80% of the substrate have been converted (Fig. 2). With porcine pepsin 60% of the leucine appears in the products Leu-Leu and Leu-Leu-Leu at about the same stage of the reaction. The high yield indicates a relatively stable intermediate. Since all the evidence so far indicates that only carboxyl groups are involved in catalysis the acyl intermediate is probably an anhydride between the leucyl group and a carboxyl group on the enzyme. The

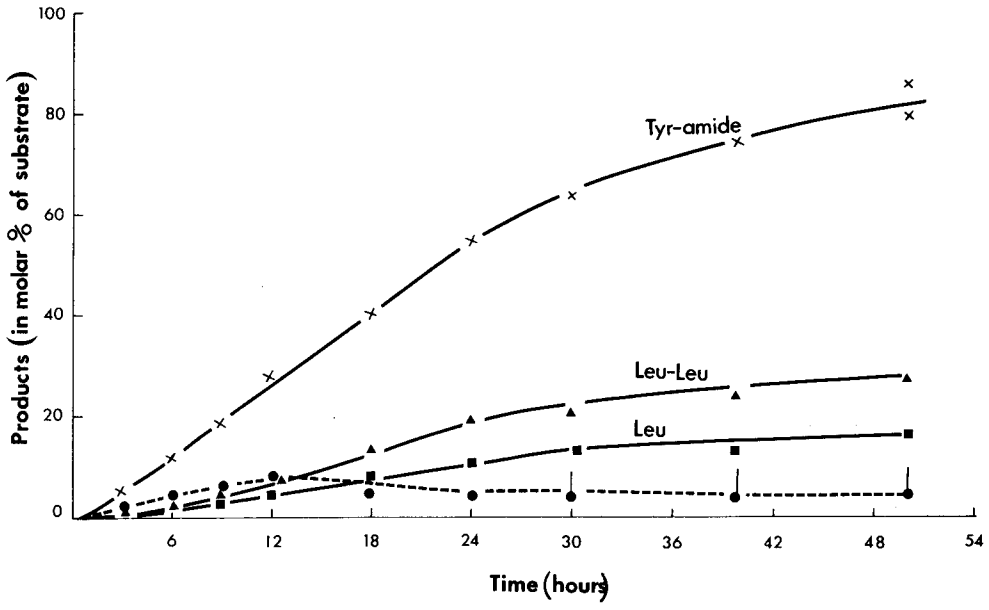


Figure 2. Action of penicillopepsin on Leu-Tyr amide.

Conditions: Leu-Tyr amide (2 μmoles) was incubated with penicillopepsin (0.6 mg) in 1 ml 0.05 M sodium citrate pH 3.4. Products were determined by the amino acid analyzer. The formation of Leu-Leu-Tyr amide was shown in a separate experiment in which the products were separated by high voltage electrophoresis. The dotted line was obtained by subtracting the sum of Leu-Leu and leucine from the Tyr-amide. It is assumed that this represents the intermediate Leu-Leu-Tyr amide.

stability is therefore surprising and suggests a unique environment with possibly restricted access to water.

Qualitatively the action of both porcine and penicillopepsin are very similar. The differences in transpeptidation yields and the formation of Leu-Leu-Leu are probably due to differences in binding at the active site.

Further implications of the results on proposed mechanisms and further details on the specificity, pH dependence and other aspects of the acyl-transfer experiments will be published in full elsewhere. A tentative scheme which accounts for both amino- and acyl-intermediates has been proposed (11).

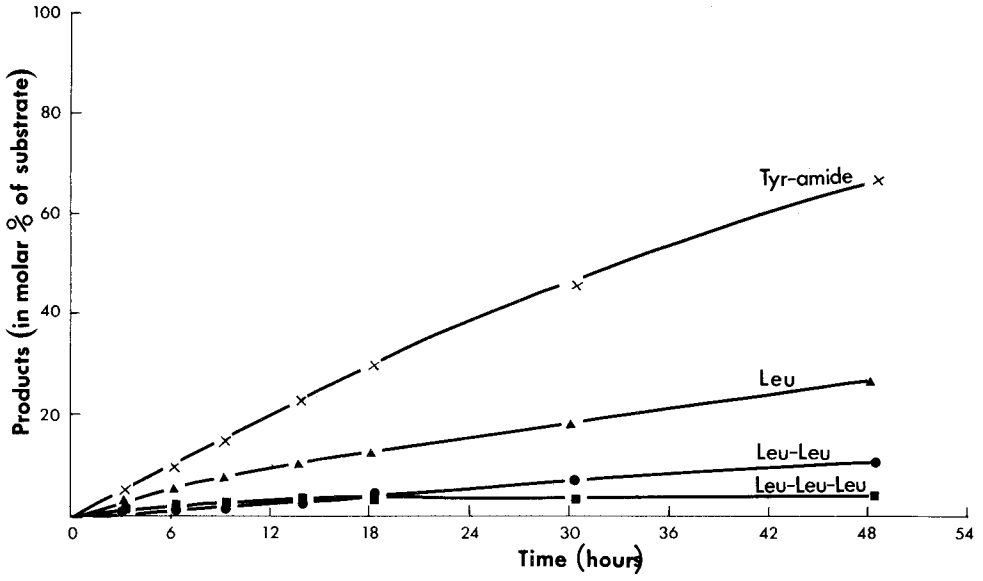


Figure 3. Action of porcine pepsin on Leu-Tyr amide.

Conditions: As for Fig. 2, but with porcine pepsin replacing penicillopepsin.

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