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Chymotrypsin-Catalysed Transpeptidations

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The transferase activity of the proteolytic enzyme trypsin has recently been investigated (see preceding paper, Waley & Watson, 1954); this work has now been extended to chymotrypsin. The substrates used were tyrosine amide, phenylalanine amide, tyrosyltyrosine amide, phenylalanylphenylalanine amide, tyrosylphenylalanine amide, phenylalanyltyrosine amide, and tyrosyltyrosine. Most of these substrates were originally investigated by Fruton & Bergmann (1942), who implicitly assumed that hydrolysis was the sole chymotrypsin-catalysed reaction.

Hydrolysis is indeed the final result, but we have shown chromatographically that transpeptidation also occurs. Moreover, the initial reaction may be entirely transpeptidation, with subsequent hydrolysis of the intermediates so formed.

EXPERIMENTAL

General. Crystalline chymotrypsin (not more than 50% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and crystalline pepsin were obtained from Armour Laboratories Ltd., Lindsey Street, London, E.C. 1. Papain (Hopkin & Williams Ltd.) was purified as described by Grassmann (1935). Ficin (L. Light and Co. Ltd.) was used as supplied. Microanalyses were carried out by Mr G. Ingram of these laboratories. Melting points are uncorrected. The error of the optical rotations is the standard deviation (Dean & Dixon, 1951). The amino acids used all had the L configuration.

Materials

Tyrosine amide. Tyrosine methyl ester (Fischer & Schrauth, 1907) was allowed to stand for 48 hr. in methanol saturated with NH_3 . After evaporation of the solution, the residue was recrystallized from ethanol (yield, 82%). After

two further recrystallizations the tyrosine amide had m.p. 153–154°; $[\alpha]_D^{20} + 19.2 \pm 0.1^\circ$ in water (c, 4.1). Koenigs & Mylo (1908) give m.p. 153–154°; $[\alpha]_D^{20} + 19.5^\circ$. (Found: C, 60.3; H, 7.0; N, 15.6; equiv. wt. 180. Calc. for $\text{C}_9\text{H}_{11}\text{O}_2\text{N}_2$: C, 60.0; H, 6.7; N, 15.5%; equiv. wt. 180.)

N-Benzoyloxycarbonyltyrosine methyl ester. The method of Bergmann & Zervas (1932) was applied to tyrosine methyl ester. After recrystallization from diisopropyl ether, N-benzoyloxycarbonyltyrosine methyl ester had m.p. 94–95°; $[\alpha]_D^{20} - 6.1 \pm 0.1^\circ$ in ethanol (c, 2.0). (Found: C, 66.0; H, 5.9. $\text{C}_{18}\text{H}_{19}\text{O}_6\text{N}$ requires C, 65.6; H, 5.8%.)

O-Acetyl-N-benzoyloxycarbonyltyrosine. N-Benzoyloxycarbonyltyrosine methyl ester was hydrolysed and acetylated by the method of Bergmann, Zervas, Salzmann & Schleich (1934). The crude product, isolated by extraction with ethyl acetate, was recrystallized from diisopropyl ether. O-Acetyl-N-benzoyloxycarbonyltyrosine had m.p. 112–113°; $[\alpha]_D^{20} + 3.2 \pm 0.2^\circ$ in ethanol (c, 2.3). Bergmann, Zervas, Salzmann & Schleich (1934) give m.p. 120–121° (not sharp); the m.p. is variable unless the specimen is carefully dried. (Found: C, 63.8; H, 5.2; N, 4.1. Calc. for $\text{C}_{19}\text{H}_{19}\text{O}_6\text{N}$: C, 63.9; H, 5.3; N, 3.9%.)

O-Acetyl-N-benzoyloxycarbonyltyrosyltyrosine amide. The peptide synthesis of Wieland & Sehring (1950) was used. Benzoyl chloride (4.6 ml.) was added to O-acetyl-N-benzoyloxycarbonyltyrosine (14.3 g.) and N-ethylpiperidine (5.4 ml.) in tetrahydrofuran (50 ml.) at 0°. After 10 min. the mixture was added to tyrosine amide (7.2 g.) suspended in ethyl acetate (50 ml.) and 1.3N- KHCO_3 (50 ml.). The mixture was stirred for 1 hr., and the product collected, washed with water and ether and recrystallized from methanol. O-Acetyl-N-benzoyloxycarbonyltyrosyltyrosine amide (10.7 g., 51%) had m.p. 209–211°; $[\alpha]_D^{20} - 14.9 \pm 0.2^\circ$ in dioxan (c, 2.4). (Found: C, 64.8; H, 5.7; N, 8.1. $\text{C}_{28}\text{H}_{33}\text{O}_7\text{N}_3$ requires C, 64.7; H, 5.6; N, 8.1%.)

Tyrosyltyrosine amide. O-Acetyl-N-benzoyloxycarbonyltyrosyltyrosine amide was suspended in 0.2N methanolic HCl and hydrogenated using 10% Pd-charcoal. The use of acid in the reduction medium removes the acetyl as well as the benzoyloxycarbonyl group (Barkdoll & Ross, 1944). The filtered solution was evaporated, the residue dissolved in the minimum amount of water and the pH brought to 7.0. The product crystallized slowly. Tyrosyltyrosine amide had m.p. 193–194°; $[\alpha]_D^{20} + 4.7 \pm 0.6^\circ$ in methanol (c, 1.7).

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(Found: C, 63.4; H, 6.2; N, 11.9; equiv. wt. 348. $C_{18}H_{21}O_4N_3$ requires C, 63.0; H, 6.2; N, 12.2%; equiv. wt. 343.)

Phenylalanine amide. Phenylalanine methyl ester (Flowers & Reith, 1953) was converted into the amide as described for tyrosine amide. After recrystallization from ethyl acetate, phenylalanine amide had m.p. 93–94°; $[\alpha]_D^{20} + 20.7 \pm 0.2^\circ$ in water (c, 1.75).

N-Benzoyloxycarbonylphenylalanylphenylalanine amide. *N*-Benzoyloxycarbonylphenylalanine (Bergmann, Zervas, Rinke & Schleich, 1934) was condensed with phenylalanine amide by the method described above. *N*-Benzoyloxycarbonylphenylalanylphenylalanine amide had m.p. 228–229°. Fruton & Bergmann (1942) give m.p. 230°. (Found: C, 70.3; H, 6.2; N, 9.5. Calc. for $C_{28}H_{27}O_4N_3$: C, 70.1; H, 6.1; N, 9.4%.)

O-Acetyl-N-benzoyloxycarbonyltyrosylphenylalanine amide. *O*-Acetyl-*N*-benzoyloxycarbonyltyrosine was condensed with phenylalanine amide as described above, and the product recrystallized from dioxan. *O*-Acetyl-*N*-benzoyloxycarbonyltyrosylphenylalanine amide had m.p. 225–226°. (Found: C, 66.8; H, 5.8; N, 8.4. $C_{28}H_{26}O_6N_3$ requires C, 66.7; H, 5.8; N, 8.3%.)

Tyrosylphenylalanine amide. *O*-Acetyl-*N*-benzoyloxycarbonyltyrosylphenylalanine amide was suspended in 0.2*N* methanolic HCl and hydrogenated using 10% Pd-charcoal. Neither tyrosylphenylalanine amide hydrochloride, nor the free base, could be purified but they were chromatographically homogeneous, and gave the expected products on acid and enzymic hydrolysis.

N-Benzoyloxycarbonylphenylalanyltyrosine amide. *N*-Benzoyloxycarbonylphenylalanine was condensed with tyrosine amide and the product recrystallized from methanol. *N*-Benzoyloxycarbonylphenylalanyltyrosine amide had m.p. 220–221°. Fruton & Bergmann (1942) give m.p. 221°. (Found: C, 67.6; H, 6.0; N, 9.3. Calc. for $C_{28}H_{27}O_5N_3$: C, 67.7; H, 5.9; N, 9.1%.)

Enzymic reactions

In the enzymic reactions, carried out at 25.8°, the substrate concentration was normally 0.05*M* and the enzyme concentration was 2%. The reactions were followed by transferring 10 μ l. samples at various times to positions on the starting line of a paper chromatogram. Whatman no. 1

paper which had been previously treated with 2*N* acetic acid, was used. The chromatographic solvents were (by vol.): *n*-butanol:acetic acid:pyridine:water (30:6:20:24; Waley & Watson, 1953); pyridine:*iso*amyl alcohol:water (7:7:6; Edman, 1946) and pyridine:*iso*amyl alcohol:water (37:43:20). The R_F values of the compounds used in this work are given in Table 1. Tyrosine-containing compounds were revealed by spraying with the Pauly reagent (Block, Le Strange & Zweig, 1952). The two-dimensional chromatograms were run on Whatman no. 3MM paper, developed first with *n*-butanol saturated with water and then with pyridine:*iso*amyl alcohol:water (Edman, 1946). It was found that the paper could be sprayed first with Pauly reagent and the spots outlined. After drying, the other side was sprayed with a solution of ninhydrin (0.4%) and cobalt chloride (0.2%) in *isopropanol* (Wiggins & Williams, 1952).

RESULTS

The following abbreviations are used: Tyr for tyrosine and tyrosyl residues, Phe for phenylalanine and phenylalanyl residues, and Am for amide groups.

Chymotrypsin

Tyrosyltyrosine amide. The course of the reaction is shown in Fig. 1.

The products are tyrosine, tyrosyltyrosine, and tyrosine amide; thus both the peptide and the amide bonds are split:

Equimolecular amounts of tyrosine and tyrosine amide should therefore be formed. It appears, however, that less tyrosine is formed; it seems possible that a reaction of the following type may occur.

The tyrosine amide formed in reactions 1 and 3 will react further as described below.

Table 1. R_F values of reference compounds

The compounds were chromatographed on Whatman no. 1 paper.

Substance	Solvent			
	Pyridine: <i>iso</i> amyl alcohol:water (7:7:6, v/v)	Pyridine: <i>iso</i> amyl alcohol:water (37:43:20, v/v)	<i>n</i> -Butanol:acetic acid:pyridine:water (30:6:20:24, v/v)	<i>n</i> -Butanol saturated with water
Tyr	0.47	0.30	0.55	0.21
Tyr.Am	0.62	0.53	0.64	0.27
Tyr.Tyr	0.64	0.47	0.76	0.20
Tyr.Tyr.Am	0.81	0.75	0.80	—
Tyr.Tyr.Tyr	0.71	—	0.86	—
Phe	0.51	—	0.65	0.33
Phe.Am	0.68	—	0.72	0.40
Phe.Phe	0.64	—	0.84	0.52
Phe.Phe.Am	0.86	—	0.86	0.69
Tyr.Phe	0.65	—	0.81	0.47
Tyr.Phe.Am	0.81	—	0.89	0.56
Phe.Tyr	0.56	—	0.82	0.37
Phe.Tyr.Am	0.82	—	0.85	0.62

Phenylalanylphenylalanine amide (Fruton & Bergmann, 1942) reacted similarly.

Tyrosine amide. The course of the reaction is shown in Fig. 2.

The products are tyrosine and tyrosyltyrosine. The tyrosine may arise by direct hydrolysis.

The tyrosyltyrosine is probably formed by the hydrolysis of tyrosyltyrosine amide (reaction 2) which may arise by transpeptidation from tyrosine amide.

It appears from Fig. 2 that initially more tyrosine than tyrosyltyrosine is formed, but that later the relative amounts are reversed. This could be caused by the occurrence of reaction 3. The tyrosyltyrosine amide postulated as an intermediate is so reactive in the presence of the enzyme, that its stationary concentration is very low.

Chymotrypsin shows the usual pH optimum of about pH 8 in this reaction.

The final product of the decomposition of tyrosine amide is tyrosine, since tyrosyltyrosine is slowly hydrolysed, as shown below.

Phenylalanine amide gave analogous results to those obtained with tyrosine amide, but reacted more slowly.

Tyrosyltyrosine (Bergmann, Zervas, Salzmann & Schleich, 1934). The main reaction here appears to be hydrolysis, which is comparatively slow:

Using a high initial substrate concentration (6%), tyrosyltyrosyltyrosine is also formed.

This was identified by comparing its R_F value with that of one of the products obtained by refluxing poly-(*O*-acetyl)tyrosine for 15 min. with constant b.p. hydrobromic acid (Abbott & Waley, unpublished work).

Tyrosylphenylalanine amide. The enzymic reaction was carried out in 30% (v/v) aqueous methanol. The main products were tyrosine and phenylalanine amide. Phenylalanine and tyrosylphenylalanine appeared later:

Phenylalanyltyrosine amide (Fruton & Bergmann, 1942). This reaction was also carried out in 30% methanol. The main products were formed as follows:

The tyrosine amide also gave the usual decomposition products (see above).

Fig. 1. Chromatogram showing the action of chymotrypsin on tyrosyltyrosine amide. Chymotrypsin (1 mg.) was added to tyrosyltyrosine amide hydrochloride (9.5 mg.) in phosphate buffer, pH 7.8 (Clark & Lubs, 1916) (0.225 ml.) and *n*-NaOH (0.025 ml.). This gives a super-saturated solution of tyrosyltyrosine amide. The chromatographic solvent was pyridine:isoamyl alcohol:water (37:43:20, v/v).

Fig. 2. Chromatogram showing the action of chymotrypsin on tyrosine amide. Chymotrypsin (20 mg.) was added to tyrosine amide (9.1 mg.) in phosphate buffer, pH 7.8 (Clark & Lubs, 1916) (1.0 ml.). The chromatographic solvent used was *n*-butanol:acetic acid:pyridine:water (30:6:20:24, v/v).

Tyrosine amide and phenylalanine amide. Equimolecular amounts of the two substrates were used. The products, identified by two-dimensional chromatography, were tyrosine, phenylalanine, tyrosyltyrosine, phenylalanylphenylalanine and phenylalanyltyrosine. The last might arise by any of reactions 13, 14 or 16:

Pepsin, papain, and ficin

Pepsin. Tyrosine amide and tyrosyltyrosine amide were not decomposed by pepsin either at pH 2.2 or at pH 4.2.

Papain. The enzyme was activated by the presence in the reaction mixture of 0.3% (v/v) thioglycolic acid. Tyrosine amide was decomposed more slowly by papain than by chymotrypsin, but gave the same products. Relatively more tyrosyltyrosine was formed at pH 7.0 than at pH 5.0. A relative increase in the extent of transpeptidation with increase in pH has been found for papain by Fruton, Johnston & Fried (1951). Tyrosyltyrosine amide gave only tyrosyltyrosine at pH 7.0, but at pH 5.0 a little tyrosine was also formed. Phenylalanylphenylalanine amide behaved similarly. Tyrosylphenylalanine amide and phenylalanyltyrosine amide each gave only one product at pH 7.0; these were assumed (by analogy with the tyrosyltyrosine amide reaction) to be the corresponding dipeptides.

Ficin. Ficin at pH 4.5 decomposed tyrosine amide and tyrosyltyrosine amide to give the same products as these substrates gave with papain, only more slowly.

DISCUSSION

The action of chymotrypsin on most of these substrates is evidently more complicated than was assumed by Fruton & Bergmann (1942). Measurement of the overall rate of hydrolysis is not adequate to give a correct picture of the course of a reaction when several reactions are proceeding simultaneously. The final products are, however, those expected to arise by hydrolysis of the susceptible bonds.

The transpeptidation reactions catalysed by chymotrypsin are remarkably similar in general outline to those catalysed by trypsin; in both cases cyclic reactions occur. Most of the products initially formed from tyrosine amide by chymotrypsin and from lysine amide by trypsin, arise from the various modes of decomposition of the dipeptide

amides. These are in each case formed by transpeptidation of the amino acid amide (reaction 5). The main difference is that trylisine is formed, but trityrosine is not. Similarly, the action of chymotrypsin on dityrosine gives tyrosine and some trityrosine, while the action of trypsin on dilysine gives lysine, trylisine, and some tetralysine. The relative rates of transpeptidation and hydrolytic reactions differ somewhat; while dityrosine is hydrolysed in the presence of chymotrypsin, dilysine in the presence of trypsin reacts only by transpeptidation. The same difference is shown by the formation of a tetrapeptide from dilysine and a tripeptide from dityrosine. Both enzymes catalyse transpeptidations giving intermediates which are broken down by hydrolysis to the amino acids.

SUMMARY

1. The action of chymotrypsin on tyrosine amide, phenylalanine amide, tyrosyltyrosine amide, phenylalanylphenylalanine amide, tyrosylphenylalanine amide, phenylalanyltyrosine amide and tyrosyltyrosine has been studied.

2. Hydrolysis is accompanied by transpeptidation in the case of tyrosine amide, phenylalanine amide and tyrosyltyrosine. The products thus formed then break down to give the amino acids as final products.

3. The action of pepsin, papain, and ficin on some of these substrates is also briefly described.

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