A STUDY OF ENZYME-SUBSTRATE COMPOUNDS

II. Preparation of Acyl-L-valyl, D-valyl, and D-phenylalanyl Derivatives of Chymotrypsin

M. M. Botvinik, I. P. Kuranova, and L. L. Ivanov*

Khimiya Prirodnykh Soedinenii, Vol. 2, No. 2, pp. 134-138, 1966

One of the fundamental factors in the mechanism of the action of proteolytic enzymes is the formation of intermediate enzyme-substrate compounds [1-4].

An intermediate enzyme-substrate compound of chymotrypsin, carbobenzoxy-D-phenylalanylchymotrypsin in which the acylating residue was an amino acid residue, was first obtained by Botvinik and Kuranova [5] in 1962.

Table 1

Reaction of Nitrophenyl Esters of Carbobenzoxyamino Acids with Chymotrypsin

p-Nitrophenyl ester	Enzyme: substrate	Reaction time, min	pH	Acylation of chymotrypsin, %	
				ester bond (by the hydroxamic reaction	amino acid (by the ninhydrin reaction)
Carbobenzoxy-L-valine	1:5	3	6,0	20	14
Carbobenzoxy-D-valine	1:5	6	6.0	18.5	10.3
The same	1:5	6	6.0	16.8	9.2
Carbobenzoxy-D-phenylalanine [5]	1:5	5	6.5	30	26

For the purposes of further synthesis and study relating to stable enzyme-substrate derivatives of chymotrypsin even with L-antipodes of amino acids, we selected a nonspecific substrate of chymotrypsin, the p-nitrophenyl and methyl esters of acyl-L-valines, whose hydrolysis has been studied by Niemann [6]. For comparison, experiments were carried out with the p-nitrophenyl esters of carbobenzoxy-D-valine, (C^{14}-benzoyl)-DL-valine, and (C^{14}-benzoyl)-DL-phenylalanine, and also with the methyl esters of (C^{14}-benzoyl)-D-valine, (C^{14}-benzoyl)-L-valine, and (C^{14}-benzoyl)-D-phenylalanine. The benzoyl derivative of the methyl esters of the amino acids containing C^{14} were obtained from benzoic acid labelled with C^{14} and the methyl esters of the amino acids by the mixed anhydride method; the nitrophenyl esters were synthesized by Bodanskii's method.

The reaction of the nitrophenyl esters of acyl-L- and acyl-D-valines with chymotrypsin led to the formation of fairly stable enzyme-substrate compounds. They were isolated by two methods: by precipitation with trichloroacetic acid or with acetone in an acid medium. In the latter case, a water-soluble sample was obtained. The content of enzyme-substrate compound in these preparations was determined as in the preceding investigation [5]: with the aid of the hydroxamic reaction and by the alkaline hydrolysis of the ester bond formed with subsequent determination of the acyl amino acid (Table 1).

In the experiments with the methyl esters of (C^{14}-benzoyl)-D-phenylalanine, (C^{14}-benzoyl)-L-valine, and (C^{14}-benzoyl)-D-valine it was found that only the first two compounds give the corresponding enzyme-substrate compounds (Table 2). The experiments with the methyl ester of (C^{14}-benzoyl)-D-valine may be considered as control experiments enabling the completeness of the removal of the absorbed impurities by washing to be estimated, which is necessary for an accurate determination of the degree of acylation of the chymotrypsin.

Thus, on nonspecific substrates, a selective capacity of chymotrypsin for reaction appears. The methyl ester of C^{14}-benzoyl)-D-phenylalanine, which contains an aromatic residue, reacts with chymotrypsin while the methyl ester

*Given at the First All-Union Biochemical Congress, 25 January 1964 (Leningrad).

of (C^{14}-benzoyl)-D-valine does not. However, replacing the methyl residue in the ester by a p-nitrophenyl residue destroys the influence of the aromatic group: the nitrophenyl ester of carbobenzoxy-D-valine forms an enzyme-substrate compound with chymotrypsin in a similar manner to carbobenzoxy-L-valine and carbobenzoxy-D-phenylalanine. The difference between them is only of a quantitative nature.

Table 2

Reaction of Esters of C^{14}-Benzoyl Amino Acids with Chymotrypsin

Radioactive substrate, counts/min per 1 mg	Time, min	Radioactivity of the enzyme substrate, counts/min per 5 mg	Yield of enzyme substrate, %
NPE of C^{14}-benzoyl-DL-phenylalanine*			
185 000	8	3883	27
NPE of C^{14}-benzoyl-DL-valine			
236 000	10	3240	20
180 000	10	2153	18
Methyl ester of C^{14}-benzoyl-D-phenylalanine**			
345 000	5	124	0.7
350 000	10	192	1
350 000	60	294	1.18
ME of C^{14}-benzoyl-L-valine			
320 000	5	488	2**
520 000	10	780	2.96**
	10	670	2.79***
	60	436	1.82***
	10	390	1.62***
	60		
ME of C^{14}-benzoyl-D-valine			
371 600	10	2	0
	60	7	0
	24 hrs	13	0

* Mean of 5 experiments

** Precipitated with TCA

*** Precipitated with acetone.

Experimental

The hydrochlorides of the methyl esters of D-phenylalanine and of L- and D-valines were obtained by Brenner's method by the action of thionyl chloride on a methanolic suspension of the corresponding amino acids.

Hydrochloride of the methyl ester of L-valine. Mp 166°–167° C, $[\alpha]_D^{19}$ + 15.45° (c 2; water). Literature data: mp 167.5°–168° C, $[\alpha]_D$ + 15.5° (c 2; water) [9], $[\alpha]_D$ + 14° (c 2; water [8].

Hydrochloride of the methyl ester of D-valine. Mp 165°–167° C, $[\alpha]_D^{19}$ −17.1° (c 2; water. Literature data: mp 167.5°–168° C, $[\alpha]_D$ −15.6° (c 2; water [9].

Hydrochloride of the methyl ester of D-phenylalanine. Mp 125°–126° C, $[\alpha]_D^{20}$ + 3.8° (c 2.67; water). Literature data: $[\alpha]_D^{25}$ + 3.9° (c 2.67; water) [10].

Synthesis of the methyl esters. Methyl ester of benzoyl-D-valine. To 0.13 g (1.065 μmole) of benzoic acid in 1 ml of absolute chloroform was added 0.15 ml of absolute triethylamine and, at −7° to −15° C, 0.15 ml (1.08 μmole) of butyl chlorocarbonate. After 30 min (−7° to −15° C), a solution of 0.18 g (1.06 μmole) of the hydrochloride of the methyl ester of D-valine in 1 ml of absolute chloroform and 0.15 ml of triethylamine were added. The reaction mixture was kept for 1 hr at −7° to −15° C and for 12 hr at 20° C. Then it was diluted with an equal volume of chloroform and was washed with 5% sodium hydrogen carbonate solution, with water, with 1 N hydrochloric acid, and with water to pH 5, and was then evaporated under vacuum; the residue was dried by repeated distillation with benzene. The residue consisted of the methyl ester of benzoyl-D-valine in the form of an oil which crystallized on trituration with petroleum ether. Yield 0.17 g (46%); mp 108°–109° C, $[\alpha]_D^{19}$ −42.6° (c 1.7; chloroform). Literature data: $[\alpha]_D^{18.5}$ −43° (c 1.17; chloroform) [11].

Methyl ester of benzoyl-L-valine. This compound was obtained in a similar manner to the preceding one. Yield

0.123 g (50%), mp 107°–109° C, $[\alpha]_D^{19}$ + 45.6° (c 0.4; chloroform). Literature data: mp 110.5°–111° C, $[\alpha]_D^{25}$ +46.0° (c 0.4; chloroform) [12] and +46.6° (c 0.4; chloroform) [13].

Methyl ester of benzoyl-D phenylalanine. The reaction was performed in a similar manner to that described above. The oily residue after the evaporation of the reaction mixture was triturated with a small amount of chloroform and was crystallized by the addition of petroleum ether. Yield 0.075 g (31%), mp 81°–81.5° C, $[\alpha]_D^{19}$ −70.62° (c 0.4; chloroform). Literature data: mp 81°–83° C [14].

p-Nitrophenyl esters. The p-nitrophenyl esters (NPE) of benzoyl-DL-valine, benzoyl-DL-phenylalanine, carbobenzoxy (CBZ)-D-valine, and CBZ-L-valine were obtained by Bodanskii's carbodiimide method [15]. The yields and melting points were: NPE of benzoyl-DL-phenylalanine 84%, 157°–158° C, literature data: mp 157°–158° C [6]; NPE of benzoyl-DL-valine 75% , 114°–115° C, literature data: mp 114°–115° C [6]; NPE of CBZ-L-valine 81%, 61° C, $[\alpha]_D^{20}$ −26° (c 2; dimethylformamide); NPE of CBZ-D-valine 82%, 62°–63° C, $[\alpha]_D^{20}$ +26.4° (c 2; dimethylformamide).

Synthesis of C^{14}-labeled compounds. The methyl esters of (C^{14}-benzoyl)-D-valine, -L-valine, and -D-phenylanine and the p-nitrophenyl esters of (C^{14}-benzoyl)-DL-valine and -DL-phenylalanine were obtained similarly by the mixed anhydride method described. The syntheses were carried out with benzoic acid having a specific radioactivity of 9.47 mC/g. The purity of the first three compounds were checked by chromatography on Whatman No. 541 paper in the butan-1-ol–2 N ammonia system and by electrophoresis on the same paper in 1/15 M phosphate buffer at pH 7.4; the purity of the NPE of (C^{14}-benzoyl)-DL-valine and -DL-phenylalanine was checked by electrophoresis. None of the compounds contained radioactive impurities.

Preparation of the enzyme-substrate compounds. Interaction of chymotrypsin with the NPE of CBZ-L- and -D-valines. A solution of 125 mg of chymotrypsin (5 μmole) in 37.5 ml of phosphate buffer with pH 6.0 was allowed to stand for 30 minutes at 37° C and was then filtered, after which 25 ml of an acetone solution of the NPE of a CBZ amino acid (25 μmole) was added. After 6 min, a 50% solution of trichloroacetic acid (TCA) was added to a final concentration of 5% (6.5 ml), and after 15 min the precipitate that had deposited was separated off and was washed with acetone and with ether. The enzyme-substrate compound was reprecipitated from a solution in 8 M urea with TCA three times, being washed each time with acetone and ether. CBZ-D- and -L-valylchymotrypsin were obtained by this method. The yields of the products were 81.5 and 80 mg, respectively. The degree of acylation of the chymotrypsin was determined as described previously [5].

Reaction of chymotrypsin with the methyl esters of C^{14}-benzoyl-D-phenylalanine, -L-valine, and -D-valine. A solution of $4–6 \times 10^{-7}$ mole of chymotrypsin in 5–7 ml of 1/15 M phosphate buffer was kept at 37° C for 30 min and was then cooled to 20°–25° C and mixed with $20–35 \times 10^{-7}$ mole of substrate dissolved in 0.5–1 ml of acetone; the reaction mixture was kept at pH 5.6–6.2 for predetermined times (see Table 2) and the enzyme-substrate compound was precipitated with trichloroacetic acid (TCA) in a similar manner to that described above, or with acetone. In the latter case, the reaction mixture was acidified with 1 N hydrochloric acid to pH 2 and the protein was precipitated by adding a tenfold volume of acetone cooled to −20° –30° C. After 15 min, the mixture was centrifuged, and the precipitate was washed with acetone (3 × 2 ml) and once with ether, and was dried in the air. For purification of the protein from the adsorbed impurities, the sample was dissolved in 2 ml of water (per 17.5 mg of the initial chymotrypsin), the solution was acidified with hydrochloric acid to pH 2, and the protein again precipitated with acetone. The purification of the enzyme-substrate compound by reprecipitation was repeated twice more. The purity of the sample was determined by the constancy of the radioactivity in the protein and in the supernatant liquid, which was practically unchanged after repeated reprecipitation.

Determination of the radioactivity of the enzyme-substrate compound and the degree of acylation. Five milligrams of the enzyme-substrate compound was placed on a steel place, moistened with acetone, and triturated to a fine paste, which was uniformly distributed over the plate and dried at 50° C. The radioactivity of the resulting samples was determined in an instrument with an end-window counter and the radioactivity of the initial substrate was determined under the same conditions (see Table 2).

M. G. Kritsman and A. S. Konikova provided us with valuable help in this work.

Summary

1. From the reaction of chymotrypsin with the NPE of CBZ-L- and CBZ-D-valine the enzyme-substrate compounds CBZ-L-valylchymotrypsin and CBZ-D-valylchymotrypsin have been isolated. The methyl esters of benzoyl-D-phenylalanine and of benzoyl-L-valine also form enzyme-substrate compounds with chymotrypsin, although to a smaller extent. The methyl ester of benzoyl-D-valine does not react with chymotrypsin.

2. Conditions for the isolation of a water-soluble enzyme-substrate compound have been found.

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7 August 1965

Lomonosov Moscow State University