AN INQUIRY INTO THE ENZYME-CATALYZED HYDROLYSIS OF LEUCINE ETHYL ESTER BY CHYMOTRYPSIN

by

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During the course of a study of the effect of ultraviolet light on the specific activity of chymotrypsin and trypsin[1], several synthetic preparations were tested for their susceptibility to digestion by crystalline beef chymotrypsin. Among others (cf. Table I), the aliphatic compound L-leucine ethyl ester (LEE) was found to undergo hydrolysis in the presence of chymotrypsin, but showed no appreciable change in the presence of trypsin (0.07 mg enzyme N/ml).

This observation seemed to merit further attention in the light of the information then at hand. WARBURG[2,3] had found that the esters of leucine are hydrolyzed by lipase-free pancreatic extracts. BERGMANN AND FRUTON[4,5] had, at a later date, demonstrated that chymotrypsin specifically attacks peptide linkages involving the carbonyl group of the aromatic amino acids, but not of leucine**. It thus appeared that our preparation of crystalline chymotrypsin might be contaminated with a leucine esterase (or peptidase) which was responsible for the observed hydrolysis of LEE. Since, however, chymotrypsin catalyzes the hydrolysis of ester analogues of its specific amide substrates, and at a much faster rate[7], it was thought possible that the esters of some of the non-aromatic amino acids might also be subject to enzymatic attack, though at a reduced rate. Experiments were accordingly designed to explore this possibility with LEE.

EXPERIMENTAL

Enzymes

Once and six-times crystallized chymotrypsin, and twice-crystallized trypsin (according to M. KUNITZ), were supplied by the Worthington Biochemical Laboratory, Freehold, N.J. The concentrations of stock enzyme solutions, prepared with hydrochloric acid as solvent, were determined spectrophotometrically[8].

Substrates

Ethyl chloroacetate, methyl dichloroacetate, and glycine ethyl ester hydrochloride were commercial preparations and were used without further purification.

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** Data was presented in a subsequent paper[6] to indicate that chymotrypsin slowly attacks the glycinamide linkage in the compounds glycylglycinamide and tyrosylglycinamide. We have not noted any enzymatic action on glycine derivatives, however, under the conditions observed (Table I).

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The sample of succinylglycine dimethyl ester[9] eas kindly furnished by Dr H. WERBIN.

L-Tyrosine ethyl ester hydrochloride, L-phenylalanine ethyl ester hydrochloride, DL-phenylalanine ethyl ester hydrochloride, L-leucine ethyl ester hydrochloride, and L-arginine methyl ester dihydrochloride were prepared in the usual manner from analytically pure amino acids.

Carbonylbisglycine ethyl ester was synthesized from phosgene and glycine ethyl ester[10].

Ethyl L-β-phenyllactate was derived from L-β-phenyllactic acid[11] by esterification with anhydrous HCl as catalyst; on recrystallization from aqueous alcohol, it gave a melting point of 46–47°C, as reported[12].

The preparation of chloroacetyl-DL-phenylalanine ethyl ester has been described elsewhere[1], as has the ethyl ester, hydrazide, and thio ethyl ester of acetyl-DL-phenylalanine[13].

Enzymatic Measurements

Hydrazidase activity. Phosphomolybdic acid reagent was used to analyze for small quantities of hydrazine resulting from hydrolysis of the corresponding acylamino acid hydrazide. This method is based on the observation that certain hydrazides do not readily reduce phosphomolybdic acid, while hydrazine does so with some facility.

The reagent was prepared as follows: 50 ml of distilled water were added with whirling to a 100 ml volumetric flask containing 10 g of sodium molybdate (dihydrate) and 1 g of sodium tungstate (dihydrate). After solution was effected, 20 ml of syrupy phosphoric acid were poured in slowly with shaking. The flask was cooled under tap water; the contents were diluted to mark with distilled water, mixed, and stored in an amber bottle.

To carry out the test, *c.* 0.05 ml (1–2 drops) of the test solution was added to 0.5 ml of phosphomolybdic acid in a small test tube. The tube was shaken briskly. Development of a distinct blue colour within 3–4 minutes indicated the presence of a minimum of 1 gamma of hydrazine. Thus 1–2% hydrolysis of a 0.03–0.05 *M* substrate solution could be readily detected. The conventional titrimetric procedures are incapable of such sensitivity.

Esterase activity. The esters studied were tested qualitatively for their sensitivity to enzymatic action by following the pH of the enzyme-substrate mixtures in 0.02 *M* phosphate buffer, initial pH 7.4–7.8, with a model G Beckman pH meter over a period of 20 min. As hydrolysis ensues, the pH drops. A weak buffer was used in these studies to maintain reasonable stability in pH. Blanks run in the absence of enzyme to detect spontaneous hydrolysis of the ester substrates were negative in all cases.

The action of chymotrypsin on LEE was followed in our early experiments with the null point potentiometric titration method of SCHWERT *et al*[7]. Subsequently it was found convenient to employ the procedure of HESTRIN[14], which involves terminating enzymatic activity after a given period of incubation by the addition of alkaline hydroxylamine reagent. Hydrochloric acid and then ferric chloride solution are pipetted in, whereupon a reddish-brown colour, due to ferric-hydroxamide complex, is produced whose intensity is determined at 520–540 mμ. For these experiments a Klett-Summerson colorimeter with a 54 filter was used. Our working procedure was as follows: 0.5 ml of 0.48 *M* phosphate buffer was mixed with 0.5 ml of 0.024 *M* LEE (adjusted to *c.* pH 7) and equilibrated to 25°C. Chymotrypsin, 0.2 ml, was added. The final concentration of LEE in the test solution was 0.01 *M* (buffer, 0.2 *M*). After a given time (*e.g.* 16 min) 2 ml of alkaline hydroxylamine were pipetted in; the mixture was shaken and permitted to stand for 2 min at room temperature. Hydrochloric acid (1 ml) and 1 ml of 0.7 *M* ferric chloride solution were then added in the usual manner.

RESULTS AND DISCUSSION

To determine the range of action of chymotrypsin, a survey was made of the extent to which alterations could be made in substrates for the enzyme without completely eliminating their susceptibility to hydrolysis. The results are summarized in Table I[15].

Although the slow hydrolysis of acetyl-DL-phenylalanine hydrazide was not entirely unexpected, the rather surprising observation was made that LEE and ethyl L-β-phenyllactate are slowly hydrolyzed by chymotrypsin. The action of chymotrypsin on the methyl ester of L-β-phenyllactic acid has since been reported by another group[16]. L-Phenylalanine ethyl ester, L-tyrosine ethyl ester, acetyl-DL-phenylalanine ethyl ester, acetyl-DL-phenylalanine thio ethyl ester, and chloroacetyl-DL-phenylalanine ethyl ester are subject to more rapid enzymatic attack.

With LEE it was found that solutions which are 0.025 *M* in substrate and contain less than 1.2 mg/ml of chymotrypsin manifest a velocity of hydrolysis that remains

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TABLE I

SUSCEPTIBILITY OF VARIOUS COMPOUNDS TO CHYMOTRYPTIC ACTION[a]

Substrate, 0.003 *M*. Temperature, 25°. pH 7.4–7.8

Substrate	Protein N per ml test solution (mg)	Activity
Acetyl-DL-phenylalanine hydrazide[b]	0.50	+
Acetyl-DL-phenylalanine thio ethyl ester[c]	0.05	+
Acetyl-DL-phenylalanine ethyl ester[c]	0.03	+
Chloroacetyl-DL-phenylalanine ethyl ester[c]	0.03	+
L-Tyrosine ethyl ester	0.06	+
L-Phenylalanine ethyl ester	0.03	+
DL-Phenylalanine ethyl ester	0.05	+[d]
Ethyl L-β-phenyllactate[c]	0.09	+
L-Leucine ethyl ester[e]	0.09	+
L-Arginine methyl ester[f]	0.05	—
Glycine ethyl ester	0.04	—
Ethyl chloroacetate[c]	0.03	—
Methyl dichloroacetate[c]	0.03	—
Succinylglycine dimethyl ester[c]	0.03	—
Carbonylbisglycine ethyl ester[c]	0.03	—

[a] Test period for esters, 20 minutes.
[b] Incubated at pH 7.4 for 14 hours. Substrate-enzyme blanks were negative.
[c] Determination carried out in 50 volumes per cent ethanol.
[d] pH 6.4.
[e] Not attacked by trypsin (0.07 mg N per ml).
[f] Very rapidly hydrolyzed by trypsin (0.02 mg N per ml).

constant for about the first 25–30% of hydrolysis. The effect of p_H on the system was measured both potentiometrically and colorimetrically, as indicated in Fig. 1. The first method reveals an optimum at p_H 6.8, while the latter reflects a shift of optimal activity of about 0.4–0.5 p_H unit towards the alkaline range.

To eliminate the possibility that the hydrolysis of LEE is caused by a contaminating leucine esterase (peptidase), the following experiments were performed:

1. LEE was incubated with six-times crystallized chymotrypsin. Hydrolysis proceeded at the expected rate.

2. LEE was incubated at p_H 7.0 with chymotrypsinogen (1.0 mg/ml). No action was noted. On the addition of a small amount of chymotrypsin, however, the p_H began to fall.

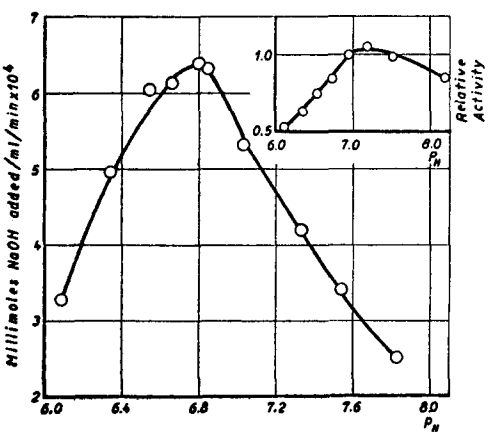

Fig. 1. p_H dependence of the hydrolysis of LEE by chymotrypsin at 25°C. Large curve refers to base initially required to compensate for acid liberated at the given mean test p_H. The 5 ml incubation mixture contained LEE, 0.025 *M*; chymotrypsin, 1.01 mg/ml; and added alkali or acid for p_H adjustment. Inset plot denotes gross decrease in residual ester colour value after 16 min incubation, relative to the decrease at p_H 6.95, here assigned a base value of 1. LEE, 0.01 *M*; chymotrypsin, 0.36 mg/ml; phosphate buffer, 0.2 *M*.

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3. To a solution containing LEE and chymotrypsinogen, p_H 7.0 was added a trace of trypsin. Within 5 minutes the p_H dropped to 6.0.

4. SMITH AND SLONIM[17] have reported that leucine aminopeptidase is activated by incubating the enzyme preparation at 40° at p_H 7.8–8.0 with 0.01 *M* manganous ion for 3 hours prior to addition to substrate. Accordingly, 1 ml of solution containing manganous sulfate (0.01 *M*) and chymotrypsin (4.75 mg/ml) at p_H 7.9 (adjustment made with dilute base—no buffer present) was kept for 3 hours at 40°, equilibrated at 25° for 30 minutes*, and then assayed against LEE**. It was found that a 58–60% loss in activity had taken place. This result is compatible with the supposition that chymotrypsin, known to be unstable under the conditions of incubation employed, is directly responsible for the hydrolysis of LEE.

To discount the possibility that LEE appears to suffer enzymatic cleavage because of contamination with an aromatic amino acid ester, the ultraviolet absorption spectrum of an aqueous solution of the LEE hydrochloride preparation was determined with a Beckman spectrophotometer. Its optical density increased continuously from 280 to 240 mμ, instead of showing specific absorption maxima characteristic of the aromatic amino acids. It is possible that trace amounts of methionine ethyl ester hydrochloride may have been present, since methionine, which is the usual impurity in crude commercial leucine, may not have been completely removed from the analytical grade leucine. The fact, however, that at least 40% hydrolysis of the ester preparation has been observed in several of our experiments speaks inevitably for the actual hydrolysis of LEE by crystalline chymotrypsin. (The LEE hydrochloride melted at 132–133° (uncorr.), in reasonable agreement with the published value of 134°[18].)

SUMMARY

1. L-Leucine ethyl ester (LEE) is hydrolyzed by pure chymotrypsin; the p_H optimum has been determined by 2 methods.

2. The ethyl esters of L-tyrosine and phenylalanine and their acyl derivatives are rapidly hydrolyzed by chymotrypsin. Acetyl-DL-phenylalanine hydrazide and ethyl L-β-phenyllactate are hydrolyzed comparatively slowly.

RÉSUMÉ

1. L'ester éthylique de la L-leucine (LEE) est hydrolysé par la chymotrypsine pure; nous avons déterminé par deux méthodes le p_H optimum de cette réaction.

2. Les esters éthyliques de la L-tyrosine et de la phénylalanine et leur dérivés contenant des restes acyl sont hydrolysés rapidement par la chymotrypsine. En comparaison l'hydrolyse de l'hydrazide de l'acétyl-DL-phénylalanine et du L-β-phényllactate d'éthyl est lente.

ZUSAMMENFASSUNG

1. Der L-Leucin-ethylester (LEE) wird durch reines Chymotrypsin hydrolysiert; das p_H-Optimum dieser Reaktion wurde mit Hilfe zweier Methoden bestimmt.

2. Die Äthylester von L-Tyrosin und Phenylalanin und ihre Acylderivate werden rasch durch Chymotrypsin hydrolysiert. Acetyl-DL-phenylalanin-hydrazid und Äthyl-L-β-phenyllactate werden vergleichsweise langsam hydrolysiert.

* At this point the p_H of the unbuffered solution was 6.8.

** Assay conducted potentiometrically in the p_H range 6.7–6.8 with 0.025 *M* substrate.

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