THE SPECIFIC ESTERASE ACTIVITY OF CARBOXYPEPTIDASE

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It was recently found that the proteolytic enzymes trypsin (1) and chymotrypsin (2) possess specific esterase activity. In addition to catalyzing the hydrolysis of specific peptides (3-7), these enzymes also catalyze the hydrolysis of those esters which possess the structural environment of the specific peptides. In order to substantiate further the suggestion of Schwert et al. (1) that the specific esterase activity is a general attribute of proteolytic enzymes, ester analogues of the specific peptide substrates for carboxypeptidase were prepared and the influence of the enzyme on these substrates was investigated. Two typical substrates for carboxypeptidase are carbobenzoxyglycyl-L-phenylalanine (8) and chloroacetyl-L-phenylalanine (8, 9). These substrates are enzymatically hydrolyzed to carbobenzoxyglycine and L-phenylalanine in the case of the former substrate, and to chloroacetate and L-phenylalanine in the case of the latter. If carboxypeptidase is endowed with specific esterase activity, the ester analogues of the above substrates, i.e. carbobenzoxyglycyl-β-phenyllactic acid and chloroacetyl-β-phenyllactic acid, should likewise be hydrolyzed under the influence of carboxypeptidase.

The carbobenzoxy group of carbobenzoxyglycyl-L-phenylalanine is required, for it has been shown that carboxypeptidase will not act on substrates which contain a free amino group in close proximity to the susceptible peptide bond (8). However, it may be expected that some

\[
\text{CH}_3
\]

\[
\text{---CO-NH---CH}_3\text{---COO---CH---CO}_2\text{H}
\]

\[
\text{Preliminary measurements have been carried out on a preparation of chloroacetyl-dl-β-phenyllactic acid which resisted all attempts of crystallization and which, as an oil, gave a neutralization equivalent of 245 (theoretical 242.5). Hydrolysis by carboxypeptidase followed zero order reaction kinetics but failed to proceed beyond 17 per cent completion. For this reason, these results have to be considered to be tentative.}
other substituent group which masks the basicity of the amino group will
serve as well. For convenience, the benzoyl derivative of glycy1-\beta-phenyl-
lactic acid was prepared (hippuryl-\beta-phenyllactic acid) instead of the corre-
sponding carbobenzoxy derivative. As it has been shown (10) that the
racemate of carbobenzoxyglycylphenylalanine is as suitable for kinetic
studies as the L isomer, the substrate used in the present investigation was
the racemic form of hippuryl-\beta-phenyllactic acid, the structure of which is
given above.

This ester is readily hydrolyzed in the presence of carboxypeptidase.
The results of quantitative kinetic studies are presented in this paper.

EXPERIMENTAL

Substrate, Hippuryl-dl-\beta-phenyllactic acid (HPLA)—The starting mate-
rials were hippuryl chloride and dl-\beta-phenyllactic acid. The preparation of
hippuryl chloride was carried out as described by Fischer (11), while that
of dl-\beta-phenyllactic acid was carried out according to the directions of
Dakin and Dudley (12). The dl-\beta-phenyllactic acid was purified by re-
crystallization from hot water. A mixture of 5.3 gm. of hippuryl chloride,
4.5 gm. of dl-\beta-phenyllactic acid, and 50 cc. of dry toluene was refluxed for
a period of 1 hour under anhydrous conditions. Upon being cooled, an
orange gummy mass separated from the solvent which was then poured off.
The reaction product was extracted with ether and the solvent was removed
by concentration in vacuo to yield an orange oil. The oil was taken up in
a hot ethanol-water mixture and treated with norit to yield a pale yellow
solution. Upon concentration in vacuo, an oil resulted which crystallized
on standing in the cold. The product was recrystallized from hot toluene,
collected, and washed with a small amount of ether. Yield, 2.0 gm.; m.p.,
121.5-122.5°.

\[ \text{C}_{13}\text{H}_{17}\text{O}_{3}\text{N} \quad (327.3). \]  
Calculated. C 66.0, H 5.24, N 4.28  
Found. " 65.9, " 5.21, " 4.38

Methods—Enzymatic measurements were carried out at 25° in the pres-
ence of 0.01 \text{M} phosphate buffer and 0.025 \text{M} \text{LiCl}. The substrate was
weighed out before each experiment, and then neutralized to pH 7.5 by
careful addition of 0.1 \text{N} \text{NaOH}. Buffer, \text{LiCl} solution, and water were
added so that after the addition of the enzyme solution the total volume
of the system would be 10 cc. The course of the reaction was followed by
direct electrometric titration as previously described (1). Enzyme solu-
tions were made up daily from a stock solution containing about 0.3 to 0.5
mg. of enzyme nitrogen per cc. The stock solution was prepared about
every 3rd day from a stock suspension of crystals (10). Enzyme nitrogen
concentrations were determined with the semimicro-Kjeldahl method.
Results

At pH 7.5, HPLA is rapidly hydrolyzed in the presence of small amounts of carboxypeptidase. Quantitative measurements revealed that the rate of hydrolysis follows zero order kinetics up to 80 per cent of the initial concentration of the enzymatically susceptible isomer of HPLA present in solutions of the dl substrate. Representative data for the hydrolysis of

![Graph showing hydrolysis of HPLA by various concentrations of carboxypeptidase](http://www.jbc.org/)

**Fig. 1.** Hydrolysis of HPLA by various concentrations of carboxypeptidase as measured by electrometric titration in 0.01 M phosphate buffer, 0.025 M LiCl, pH 7.50 at 25°. The initial concentration of dl-HPLA in all three systems was 0.010 M. The amount of carboxypeptidase N in mg. present in these systems (final volume 10 cc.) is shown by the following: O 0.00500, 0 0.00500, □ 0.00375, △ 0.00250, ▲ 0.00125, □ 0.000625. The inset graph indicates the linear relationship between the rate of hydrolysis of HPLA and the amount of carboxypeptidase present.

0.01 M solution of dl-HPLA by five different concentrations of carboxypeptidase, varied over an 8-fold range, are given in Fig. 1. The precision of the analytical method is evidenced by the close fit of the points obtained in duplicate experiments at the highest enzyme concentration. The inset graph of Fig. 1 demonstrates that the rate of hydrolysis is proportional to the enzyme concentration in the system.

Although careful examination of the analytical data excluded rates other than that of zero order, a dependence of the rate constant on initial substrate concentration became apparent. Representative data are given in
Fig. 2, in which the titration data for four different initial substrate concentrations at a constant enzyme concentration of $2.5 \times 10^{-4}$ mg. of N per cc. are plotted according to zero order kinetics. The rate of hydrolysis, represented by the slope of the linear portion of the curves, is highest for the lowest initial substrate concentration and decreases with increasing substrate concentration as shown in the inset graph of Fig. 2. The substrate concentration dependence of the reaction constant decreases mark-

![Graph](image)

**Fig. 2.** Dependence of hydrolysis of HPLA on initial substrate concentration in 0.01 M phosphate buffer, 0.025 M LiCl, pH 7.50 at 25°. The amount of carboxypeptidase N in mg. present in these systems (final volume 10 cc.) was 0.0025. The initial concentration of *dl*-HPLA is shown by the following: ■ 0.005 m, △ 0.010 m, ○ 0.015 m, ○ 0.020 m. The inset graph indicates the relationship between the rate of hydrolysis of HPLA and the initial concentration of HPLA.

edly with increasing substrate concentration and appears to vanish near 0.02 m *dl*-HPLA.

In view of the inhibition of carboxypeptidase activity toward carboxybenzoxyl-L-phenylalanine by chloroacetate (8, 9) and by *d*-phenylalanine (13), the influence of these two compounds as well as that of *dL*-phenyllactic acid on the hydrolysis of HPLA was investigated. No measurable effect was noted when 0.007 m sodium chloroacetate was added to the enzyme-substrate system containing 0.01 m *dl*-HPLA. The additions of 0.01 m *dl*-phenyllactic acid and of 0.01 m *dL*-phenyllactic acid to the same system decreased the zero rate of hydrolysis by, respectively, 38 and 17 per cent. A more detailed study of the inhibition by these two com-
pounds will be deferred until analogous experiments on their influence on the hydrolysis of carbobenzoxyglycyl-L-phenylalanine have been completed.\textsuperscript{2}

The enzymatic specificity of the hydrolysis of HPLA was ascertained by the exclusion of a measurable extent of spontaneous hydrolysis of this ester in the buffer system used, and by measurements of the effects of other pancreatic proteolytic enzymes. Of the latter, crystalline ribonuclease\textsuperscript{3} (0.3 mg. per cc.) and crystalline trypsin\textsuperscript{4} (0.1 mg. of N per cc.) had no measurable effects. Crystalline chymotrypsin\textsuperscript{4} caused a slow hydrolysis, the rate per mg. of enzyme N per cc. being about 0.005 of that produced by carboxypeptidase, \textit{i.e.} $6.2 \times 10^{-7}$ mole per minute per mg. of enzyme N for chymotrypsin compared to $1.2 \times 10^{-5}$ for carboxypeptidase.

\section*{DISCUSSION}

The present experimental data provide evidence that carboxypeptidase is capable of catalyzing the hydrolysis of a specific ester. The chemical structure of the substrate is such as to allow only one interpretation for the liberation of titratable acid during enzymatic hydrolysis, namely the splitting of the ester bond, since carboxypeptidase is incapable of hydrolyzing the secondary peptide bond of carbobenzoxyglycyl-L-phenylalanine (4).

The observed zero order kinetics for the enzymatic hydrolysis of HPLA is in accord with analogous data for the specific esterase activity of trypsin (1). This may be ascribed in both cases to full saturation of the enzyme by the substrate, the rate-determining step apparently being that of the activation on the enzyme surface. With both enzymes, too, the hydrolysis of the corresponding peptide apparently follows first order kinetics (4, 14). However, in the present case, the dependence of the rate of ester hydrolysis on initial substrate concentration requires consideration. It has been found that the apparent first order reaction constants for the hydrolysis of carbobenzoxyglycyl-m-phenylalanine by carboxypeptidase (13) and for the hydrolysis of specific peptides and esters by chymotrypsin (2) decrease with increasing substrate concentration. This dependence of first order reaction constants upon substrate concentration can be accounted for by the shift of equilibrium between combined and free enzyme with changing substrate concentration. However, as would be expected, the initial reaction velocity increases with increasing substrate concentration.

The same explanation cannot apply to the observed decrease of the reaction constant of ester hydrolysis with increasing substrate concentration. Since the ester hydrolysis follows zero order kinetics, the initial velocity,
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as well as the rate constant, decreases with increasing substrate concentration. Nor can incomplete dissociation of HPLA or of the reaction products at the pH of the electrometric titrations (pH 7.5) account for this effect. Electrostatic interactions between enzymes and substrate may conceivably be considered and will be evaluated in further experiments.

Because of the difference in order of the reaction rates, strict comparison of the specific esterase and peptidase activities of carboxypeptidase is difficult. Further limitations of such a comparison arise from the large and oppositely directed dependence of the hydrolysis rates of peptide and ester upon substrate concentration.

The specific ester substrates for trypsin and chymotrypsin that have so far been tested are limited to those which contain a terminal methyl or ethyl group. Although these esters are structural analogues of specific amide substrates for trypsin and chymotrypsin, it is doubtful that amides of this type occur in native proteins. The hydrolysis of HPLA by carboxypeptidase is a more convincing proof for the inability of proteolytic enzymes to differentiate between peptide and ester bonds, since N-substituted gly-cylphenylalanine is a natural substrate for carboxypeptidase and HPLA a true analogue thereof.

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SUMMARY

Crystalline carboxypeptidase exhibits esterase activity toward an ester analogue of a specific peptide substrate. Hippuryl-\(\beta\)-phenyllactic acid (HPLA) is hydrolyzed at a fast rate according to zero order kinetics. Quantitative studies on HPLA, including the effects of enzyme and substrate concentrations, are described.

The synthesis of hippuryl-\(dl\)-\(\beta\)-phenyllactic acid is described.

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