## Fatty acid esters as substrates for trypsin and chymotrypsin

It has been demonstrated that the proteolytic enzymes trypsin[1] and chymotrypsin[2] catalyze the hydrolysis of certain substituted amino acid esters. Even *p*-nitrophenyl acetate is hydrolyzed by chymotrypsin[3].

We now find that recrystallized trypsin and chymotrypsin hydrolyze *n*-fatty acid esters of hydroxybenzoic acids, the same substrates that have been used for the spectrophotometric assay of typical esterases such as liver esterase[4], pancreatic "ali"-esterases[5] and serum cholinesterase[6,7]. Owing to the ionized carboxyl group these esters are soluble at pH 8 and suitable for kinetic studies by means of direct spectrophotometric measurement of the increase in free hydroxybenzoic acid. Details of the method have been described previously[6,5].

The present experiments were carried out with the Na salts of esters of *ortho*- and *meta*-hydroxybenzoic acids with *n*-fatty acids of different C-chain lengths ($C_2$, $C_3$ ...). The increase in light absorption (300 m$\mu$, 1 cm) was measured at frequent time intervals with the aid of a Beckman Model D-U spectrophotometer provided with thermospacers through which water of 30° was circulated. The reference cell contained buffer (0.0375 *M* Veronal, pH 8) and substrate in the same concentration as in the reaction mixture; thus the influence of non-enzymic hydrolysis on the increase in light absorption was automatically cancelled. The following enzyme preparations were used: crystalline trypsin (Armour, lot No. 181), containing 14.1% N, 2.0% ash and 7.8% moisture; crystalline chymotrypsin (Armour, lot No. 283) containing 14.56% N, $<$ 0.1% ash and 6.24% moisture. The activity of the chymotrypsin preparation expressed as mequiv. hemoglobin-tyrosine/mg protein N/min at 35.5° was 0.0397, which is about the same as the original several times recrystallized preparations of NORTHROP *et al.*[8]. The activity of the trypsin preparation was only 50–60% that of the best preparation described by these authors*.

TABLE I

THE INFLUENCE OF ENZYME CONCENTRATION AND OF TIME ON THE ESTEROLYTIC ACTION OF CRYSTALLINE TRYPSIN AND CHYMOTRYPSIN

The amount of substrate hydrolyzed in a particular time interval (pH 8, 30°) is expressed as the increase in extinction at 300 m$\mu$ over a 1 cm light path; 1 $\mu$mole *m*-hydroxybenzoic acid liberated corresponds to a $\Delta E$ of 0.375.

| | *Trypsin, 0.02 M m-$C_6$ (mg enzyme per 3 ml)* | | | *Chymotrypsin, 0.033 M m-$C_7$ (mg enzyme per 3 ml)* | | |
|---|---|---|---|---|---|---|
| | *1.0* | *0.5* | *0.25* | *0.32* | *0.16* | *0.08* |
| 5–15 min | 0.129 | 0.067 | 0.032 | 0.172 | 0.083 | 0.041 |
| 15–25 min | 0.123 | 0.066 | 0.033 | 0.179 | 0.086 | 0.040 |
| 25–35 min | 0.115 | 0.061 | 0.031 | 0.168 | 0.084 | 0.042 |
| Total 30 min | 0.367 | 0.194 | 0.096 | 0.519 | 0.253 | 0.123 |
| × Dilution | 0.367 | 0.388 | 0.384 | 0.519 | 0.506 | 0.492 |
| % Deviation from average | −3.4 | +2.1 | +1.1 | +2.6 | 0 | −2.8 |

Data on the effect of enzyme concentration and of time on the extent of hydrolysis are presented in Table I. The activity is not strictly proportional to the enzyme concentration. Deviation from proportionality was as high as 5–6% in the case of trypsin (first and second column Table I). The reaction rate with high concentrations of trypsin (first column Table I) decreases more than 10% during 30 min. About 1.6% of the substrate is hydrolyzed during this time interval which, on the basis of substrate depletion, would cause a decrease in rate of less than 1% as can be calculated with the aid of the Michaelis constant estimated as $8.5 \cdot 10^{-3} M$ from the data in Fig. 1A. The observed decrease is probably due to autolysis of the enzyme which occurs to a greater extent at the higher trypsin concentration[9] and thus may also influence proportionality between rate and enzyme concentration. The decrease in this early stage of the reaction was nearly proportional to time; accordingly, the initial rates (Fig. 1A) were obtained by linear extrapolation to zero time. Proportionality between enzyme concentration and reaction

* Analyses by Armour and Company Research Division.

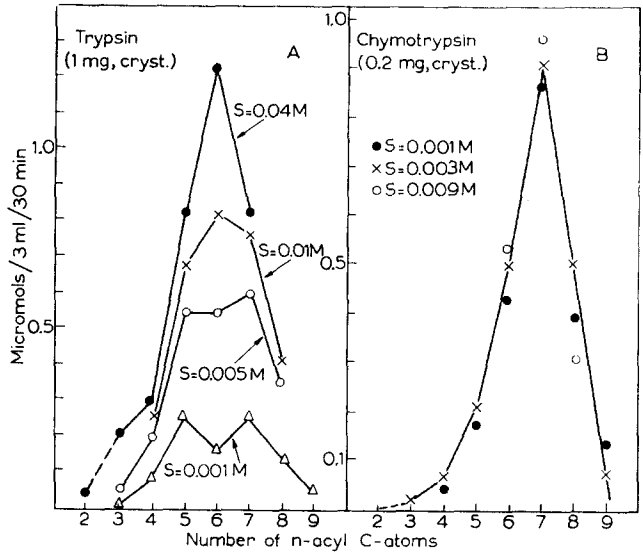


Fig. 1. A. Influence of the C-chain length of the *n*-fatty acid moiety of the substrates (esters of *m*-hydroxybenzoic acid in concentration *S*) on the rate of hydrolysis by recrystallized trypsin. B. Influence of the C-chain length of the *n*-fatty acid moiety of the substrates (esters of *m*-hydroxybenzoic acid in concentration *S*) on the rate of hydrolysis by recrystallized chymotrypsin.

rate and constancy of the rate was more perfect with chymotrypsin than with trypsin, although it would appear (Table I) that the specific activity of chymotrypsin increases slightly with increasing enzyme concentration. Nevertheless, if these deviations were ignored, the overall maximum error for the range of enzyme concentrations of Table I is less than 5 %, even in the case of trypsin. The difference between duplicate determinations at the same enzyme concentration was usually not more than 1 %, provided sufficiently large extinction increases were measured.

In view of the high enzyme concentrations that were required, the possibility exists that the observed activity is due to contaminating proteins with high esterolytic activity. However, the following observations indicate that all the esterolytic activity is due to the proteolytic enzymes.

1. The esterolytic activities of the trypsin and the chymotrypsin preparations are inhibited by soybean inhibitor (Worthington). In the case of trypsin the inhibition is proportional to the inhibitor concentration up to 90 % inhibition. In contrast, the inhibition of chymotrypsin per unit amount of inhibitor decreases with increasing inhibitor concentration. This indicates stoichiometric and irreversible binding of trypsin and an equilibrium reaction in the case of chymotrypsin. Identical observations have been reported[8] with respect to the proteolytic activity of these enzymes.

2. When an aqueous solution of the trypsin preparation (5 mg/ml) is acidified to pH 2 with HCl, brought to boiling, cooled and left overnight at room temperature, the esterolytic activity ($m$-$C_6$) is the same as that of the unboiled solution. Such stability (reversible denaturation) is a typical property of the trypsin molecule[8].

3. The esterolytic activity of the chymotrypsin preparation is inhibited by $\beta$-phenyl-propionic acid and by benzoyl-phenylalanine which are typical inhibitors of this enzyme[10]. The inhibition with $o$-$C_7$ as the substrate is much larger than in the case of $m$-$C_7$. In view of the finding that the $K_M$ of $o$-$C_7$ is of the order of 30 times larger than that of $m$-$C_7$ (see below), this indicates that these substrates compete with the inhibitors for attachment to the active center of the proteolytic enzyme.

A notable result of the present investigation is the observation that, like the above-mentioned esterases, trypsin and chymotrypsin display sharp optima of activity with respect to the number of C-atoms in the *n*-acyl moiety of the substrate as is shown in Fig. 1. Here the initial rates are given at different concentrations of the substrates below the critical concentrations of micelle formation[11]. For trypsin (Fig. 1A), the maximum is $C_6$ at high substrate concentrations but at low substrate concentrations this changes to maxima at $C_5$ and $C_7$. This is due to differences in the kinetic constants $K_M$ and $V_m$.

Chymotrypsin (Fig. 1B) shows an optimum of activity with $C_7$ as the substrate. Increasing the substrate concentration from 0.001 $M$ to 0.009 $M$ effects little increase in the rates of hydrolysis by this enzyme. In some cases ($C_8$, $C_9$) increasing the concentration results even in a decrease of the rate. In the case of $C_7$, the lowest substrate concentration ($10^{-3}$ $M$) used saturates the enzyme to the extent of about 90 %, indicating that the Michaelis constant is of the order of $10^{-4}$. This

cannot be measured accurately with the present method of activity determination, at least not on the basis of initial reaction rates. However, it was found that with the corresponding *ortho* esters the $K_M$ values are much larger and measurable. For $o$-$C_7$, a $K_M$ of $3 \cdot 10^{-3} M$ was found. The $V_m$ values of chymotrypsin with the *ortho* esters as substrates, although generally lower, show the same pattern (maximum at $C_7$) as with the *meta* esters as the substrates. A detailed report of these investigations will be given at a later date.

Based on a molecular weight of 27,000 and the assumption that each enzyme molecule carries one active group[12], the turnover number of $m$-$C_7$, calculated from $V_m$ (Fig. 1B), would be about 4/mol. chymotrypsin/min; that of trypsin with $C_6$ as the substrate would be even lower. Such turnover numbers are of a lower order of magnitude than those of esters of certain substituted amino acids but, although lower, are of the same order of magnitude as those of the amino acid amides[2,13]. It might be expected that these amino acids, *e.g.* benzoyl-arginine and benzoyl-tyrosine[14], esterified with the phenolic group of hydroxybenzoic acids, would be ideal substrates for direct spectrophotometric assay, provided such esters would be sufficiently soluble. An investigation to this effect is in progress. In the meantime it would appear that certain of the above fatty acid esters, *e.g.* $m$-$C_6$ for trypsin and $o$-$C_7$ for chymotrypsin, provide convenient substrates for direct and continuous spectrophotometric assay of these enzymes and are suitable for kinetic studies in aqueous solutions. Despite low turnover numbers the enzyme concentrations ($10^{-5}$–$10^{-6} M$) required are still negligible[15] with respect to any of the substrate concentrations needed for accurate determination of the kinetic constants on the basis of initial reaction rates.

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## The relative potencies of thyroxine and triiodothyronine analogues *in vivo*

It is well known that the activity of triiodothyronine greatly exceeds that of thyroxine in preventing thiouracil-induced goitre in rats[1,2], in raising oxygen consumption in small laboratory animals[2,3,4,5], in hastening the death of mice from anoxia[2], and in accelerating amphibian metamorphosis[6,7]. Further, it has been shown[8] that if iodine is replaced by bromine or chlorine in the thyronine molecule, the trihalogenated compounds possess, in general, a higher potency than the corresponding tetrahalogenated compounds, when assayed by the goitre prevention method. However, this is not always true; MUSSETT AND PITT-RIVERS[9] in a survey of several pairs of tetra- and trihalogenated thyronine analogues have found that in one instance this situation is reversed: 3:5-diiodo-3′:5′-dichlorothyronine is five times as active as the 3:5-diiodo-3′-chloro-derivative.

It was thought of interest to determine whether the relatively greater activity of this tetrahalogenated thyronine would also be found by another method of assay, and both compounds were tested in accelerating the metamorphosis of tadpoles of *Rana esculenta* by the method of SHELLABARGER AND GODWIN[7]. The results are given in Table I. It can be seen that the tadpole test gives the same relative potencies for these two compounds as is obtained by the goitre prevention method.