COVALENT ATTACHMENT OF CHYMOTRYPSIN TO POLY[N-(2-HYDROXYPROPYL)METHACRYLAMIDE]*

Ants LÄÄNE[a], Vladimír CHYTRÝ[b], Mati HAGA[a], Peeter SIKK[a], Aavo AAVIKSAAR[a] and Jindřich KOPEČEK[b]

[a] *Institute of Chemical Physics and Biphysics, Estonian Academy of Sciences, Tallinn 200 001, USSR and*
[b] *Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6*

Received January 11th, 1980

Chymotrypsin has been covalently bound on a water-soluble biocompatible carrier poly[N-(2-hydroxypropyl)methacrylamide] by three different methods. The catalytic activity of the polymer bound enzyme in the reaction with low-molecular substrates coincided with the activity of native chymotrypsin. The protective effect of the carrier polymer in chymotrypsin thermoinactivation at pH 8·0 was larger than at pH 6·1, particularly at higher concentrations of the enzyme (at 10^{-5}M), which indicates that the polymer protects chymotrypsin not only against irreversible thermoinactivation but also against autolysis. Significant protective effect of the carrier polymer in the interaction of the polymer bound enzyme with high-molecular soybean trypsin inhibitor has been found.

Water-soluble high molecular weight polymers may have protective effect in the inactivation of the bound enzyme[1,2], and so a significant medical use in the preparation of drugs with prolonged circulation time[3,4]. The chemical nature of a carrier can effect intrinsic properties of a polymer bound enzyme in addition to the steric and diffusional effects usually observed with such enzymes[5].

The paper reports covalent coupling of chymotrypsin on biocompatible carrier poly[N-(2-hydroxypropyl)methacrylamide] by three different methods: *A*) cyanuric chloride method, *B*) hydrazide method, *C*) active esters method. The following enzymatic properties of the preparations have been investigated: the kinetics of the reaction with acetyl-L-phenylalanine ethyl ester, the kinetics of thermoinactivation, the inhibition with low-molecular inhibitors (indole, proflavine), and with soybean trypsin inhibitor.

* Research was carried out under the bilaterial agreement on cooperation between the Czechoslovak Academy of Sciences and the Academy of Sciences of the USSR.

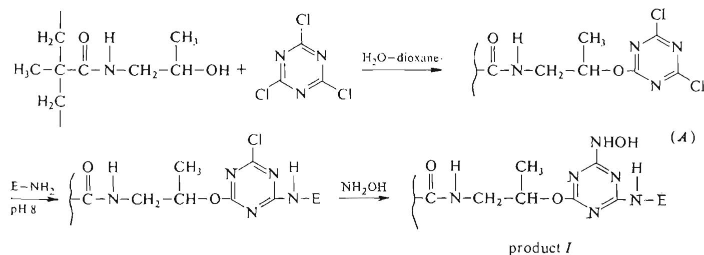
EXPERIMENTAL

Materials

Crystalline bovine α-chymotrypsin (USSR, Olaine Chemistry Plant) was used without further purification; according to the active site titration data it consisted of 75—80% of active enzyme. The active site titrant, *p*-nitrophenyl *p*-(N,N,N-trimethylammonium)cinnamate, was prepared according to Knowles and coworkers[6]. Acetyl-L-phenylalanine ethyl ester (APEE) was synthesized and purified by conventional methods for acyl amino acid ester synthesis[7]. Poly[N-(2-hydroxypropyl)methacrylamides] with various molecular weights and a copolymer of N-(2-hydroxypropyl)methacrylamide with *p*-nitrophenyl ester of methacryloylated glycylglycine (m.w. 13000) were prepared as described earlier[8,9]. Soybean trypsin inhibitor was from Olaine Chemistry Plant, USSR. All other reagents were commercial products from "Reakhim" (USSR). Sephadex G-75 and G-100 were products of Pharmacia Fine Chemicals (Uppsala, Sweden).

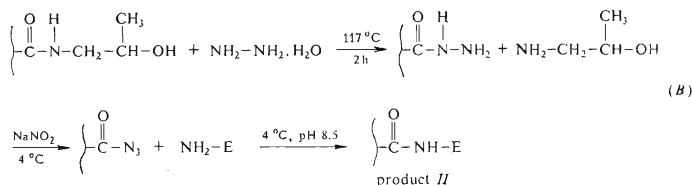
Chymotrypsin Binding Methods

A) Poly[N-(2-hydroxypropyl)methacrylamide] (1 g) of m.w. 90000 was dissolved in 25 ml water and then 25 ml cyanuric chloride solution in 1,4-dioxane was added; pH of the solution was kept 8·3 by adding $NaHCO_3$. The reaction was carried out at room temperature for half an hour. The activated polymer was removed from the solution by precipitation in acetone and washed free from unreacted cyanuric chloride with acetone. The activated polymer contained 0·91 mM of active chloride per gram of the preparation as estimated by the method of determination of amino group concentration by means of 2,4,6-trinitrobenzenesulphonic acid[10] after the treatment of polymer with hexamethylene diamine. The activated polymer (1 g) was dissolved in 25 ml 0·1M borate buffer pH 8·3, and 100 mg of α-chymotrypsin was introduced. The reaction mixture was kept overnight and then 3 ml 1M-NH_2OH solution pH 8·6 was added to remove active chlorine. After 2 hours of reaction, pH of the mixture was changed to 5·0 by adding 1·0M solution of HCl, and the products were separated on a Sephadex G-100 column. The resolution between free and polymer bound chymotrypsin on a 4·5 × 140 cm column was complete (Fig. 1). The possible course of the reaction may be in a simplified way presented by reaction (*A*) (where E—NH_2 represents the enzyme molecule).

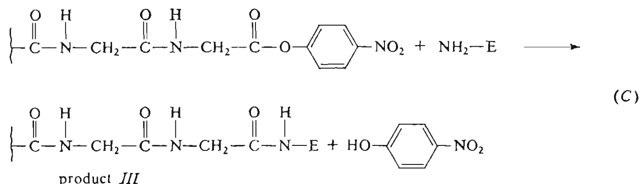


B) Poly[N-(2-hydroxypropyl)methacrylamide] (1·22 g) of m.w. 53000 was refluxed with 15 ml hydrazine hydrate for 2 h. The hydrazide of the polymer was precipitated in 300 ml acetone. The precipitate was dissolved in 5 ml water and separated on a Sephadex G-25 column. Polymer-

containing fractions were lyophilized. The polymer hydrazide (100 mg) was dissolved in 1·5 ml 0·2M-HCl at 4°C, and 0·5 ml of 10% $NaNO_2$ solution was added dropwise. Then the reaction mixture was stirred for half an hour, the solution of 50 mg sulfamic acid in 0·5 ml water and 4 ml 0·15M-$Na_2B_4O_7$ solution were added, and pH was kept 8·5 by adding 0·5M solution of NaOH. The activated polymer contained 0·19 mM of reactive groups as estimated by the method of the determination of amino group concentration after the treatment of the polymer with hexamethylene diamine[10]. α-Chymotrypsin (13 mg) was added to the solution of the activated polymer and the reaction mixture was kept at 4°C for 16 hours. The products were separated on a Sephadex G-100 column (the separation was comparable with that in Fig. 1) and lyophilized. The protein content of the preparation was about 3·5% as calculated from the absorption data at 280 nm. The possible course of the reaction may be in a simplified way presented by reaction (*B*).



C) Chymotrypsin was bound on a copolymer of N-(2-hydroxypropyl)methacrylamide with *p*-nitrophenyl ester of methacryloylated glycylglycine (m.w. 13 000). The *p*-nitrophenyl ester content was 8·0 mol%. 200 mg of the polymer was added to the solution of 130 mg α-chymotrypsin in 10^{-4}M-HCl at 25°C and pH 4·2. After 10 minutes incubation, 1M-Na_2CO_3 solution was added to raise pH to 8·5. At this pH the reaction between chymotrypsin and the active groups of the polymer was carried out. After 4 min of the reaction, 1 ml 1M ethanolamine solution pH 8·5 was added and after one more minute pH of the solution was changed to 5. The reaction mixture was fractionated on a Sephadex G-100 column with a result analogous to Fig. 1. The protein content of the product about 24% has been calculated from the absorption data at 280nm. The supposed course of the reaction may be presented by reaction (*C*), where the possible side reactions (hydrolysis and enzyme catalyzed hydrolysis) are not indicated.



Kinetic Measurements

The activity of chymotrypsin in all the products was determined as the initial rate of steady state hydrolysis of APEE at 25°C pH 8·0 in 0·1M-NaCl measured with the pH-stat method[11] on a Ra-

diometer TTT2/SBR3/ABU12 Titrigraph set (Denmark). The absolute concentrations of chymotrypsin active sites were determined by the method of Knowles and coworkers[3].

The kinetics of chymotrypsin thermoinactivation was studied at 50°C pH 6·1 and 8·0, in 0·1M-NaCl and in 0·001M sodium phosphate buffers, respectively. The enzyme was incubated in a thermostated vessel. To measure thermoinactivation rate the samples were taken, rapidly cooled to 25°C, and removed into the pH-stat cell for activity measurement.

The chymotrypsin–inhibitor complex dissociation constants K_i for indole, proflavine and soybean trypsin inhibitor were calculated from the slopes of the straight lines in the coordinates v/v_i against $[I]_0$, according to the equation

$$\frac{v}{v_i} = 1 + \frac{K_m^{app}}{K_m^{app} + [S]_0} \frac{1}{K_i} [I]_0 , \quad (1)$$

where v_i and v are the initial rates of hydrolysis of the substrate, S, in the presence and the absence of the inhibitor, I, respectively, and K_m^{app} is the apparent Michaelis constant.

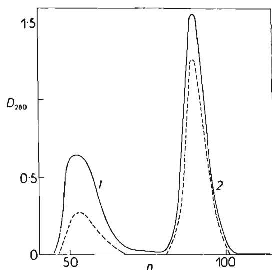


FIG. 1

Separation of Chymotrypsin Covalently Bound on Cyanuric Chloride-Activated Poly[N-(2-Hydroxypropyl)methacrylamide] by Gel Filtration on Sephadex G-100 Fine (Column 4·5 × 140 cm, fraction volume 15 ml)

Curves: ——— D_{280} ----- chymotrypsin activity, μmol APEE/ml min. Peaks: 1 polymer bound chymotrypsin (product I), 2 free chymotrypsin.

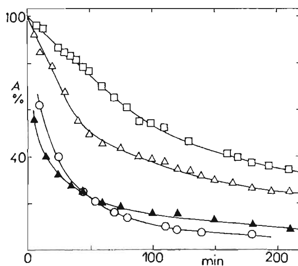


FIG. 2

Thermoinactivation of Chymotrypsin (activity A vs time) at 50°C in 0·1M-NaCl (pH 6·1, initial concentration of enzymes $[E]_0$ 1·0 . 10^{-5} mol)

○ Free enzyme; products: △ *I*, ▲ *II*, □ *III*.

RESULTS AND DISCUSSION

Reaction with Acetyl-L-phenylalanine Ethyl Ester (APEE)

The obtained pH_{opt} value for all three products (*I* to *III* in (*A*)–(*C*)) was in line with that of free enzyme. The specific activities μM APEE/min mg of the product at pH 8·0 were 0·5% for the product *I*, 1·1% for the product *II* and 23·3% for the product *III* compared to free chymotrypsin. For the reactions of products *I* and *III* with APEE at 25°C and pH 8·0, the k_{cat} values 81 s^{-1} and 79 s^{-1} were calculated from active site titration data. For native chymotrypsin at closely similar conditions, k_{cat} 68·6 s^{-1} was obtained[12]. It shows that the catalytic activity of the polymer bound enzyme in reactions with low-molecular substrates remains practically unchanged. Taking into consideration the protein content 24% in the product *III* (obtained from absorption data at 280 nm) and the specific activity of the preparation (23·3% of the specific activity of the free enzyme), it can be concluded that all molecules of the enzyme which became bound to the polymer remained active.

Apparent Michaelis constants K_m^{app} in the reaction with APEE were about 2 . 10^{-3}M for all three products in agreement with K_m^{app} 1·9 . 10^{-3}M for native chymotrypsin[12].

Thermoinactivation Kinetics

Thermoinactivation experiments at 50°C and pH 6·1 revealed some increase in the thermostability of products *I* and *III* compared with free enzyme (Fig. 2). Further studies of thermoinactivation were carried out on the product *III* as the most active and the most stable one. At pH 8·0 the difference between thermostabilities of the free and polymer bound enzymes was remarkably larger than at pH 6·1. The thermoinactivation curve at pH 8·0 can be approximated with the kinetic law for a bimolecular reaction (Fig. 3). It is in accordance with the earlier finding of Antonov and coworkers[13] that the thermoinactivation of chymotrypsin at sufficiently high enzyme concentration ($\geqq 10^{-5}$M) proceeds by the scheme

$$E_N \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_D \xrightarrow{k_2[E_N]} P_A , \qquad (D)$$

where E_N is the native enzyme, E_D is the denatured enzyme, P_A denotes products of autolysis. They also found that $k_{-1} \gg k_2[E_N]$, and the reaction proceeded by the kinetic law for a bimolecular reaction, according to

$$\frac{d[E_N]}{dt} = -\frac{k_1 k_2}{k_{-1}} [E_N]^2$$

for the rate of the change of native enzyme concentration. From the slopes of the

straight lines in the coordinates $1/A$ against t, where activity A is proportional to $[E_N]$, it was found that at pH 8·0 the stability of product *III* was 21 times and at pH 6·1 only 3 times higher than that of free chymotrypsin. The conclusion that binding to polymer protected the protein also against autolysis was drawn from these data.

TABLE I

Inhibition of Free and Immobilized Chymotrypsin at 25°C in 0·2M-KCl, pH 8·0

Method of immobilization	K_m^{app}, mol (APEE)	K_i, mol		
		soybean inhibitor	proflavine	indole
—	$1.9 \cdot 10^{-3}$	$5.7 \cdot 10^{-9}$	$7.1 \cdot 10^{-5}$	$8.0 \cdot 10^{-4}$
A	$2.0 \cdot 10^{-3}$	$3.0 \cdot 10^{-8}$	$4.9 \cdot 10^{-5}$	$8.0 \cdot 10^{-4}$
C	$2.2 \cdot 10^{-3}$	$1.6 \cdot 10^{-7}$	$6.5 \cdot 10^{-5}$	—

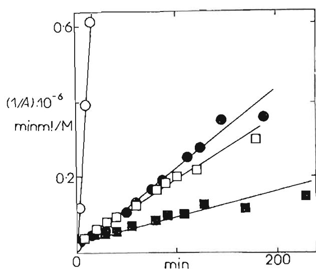


FIG. 3

Thermoinactivation of Chymotrypsin at 50°C in 0·1M-NaCl ($[E]_0 = 1.0 \cdot 10^{-5}$ mol)

Free enzyme: ○ pH 8·0, ● pH 6·1; product *III*: □ pH 8·0, ■ 6·1.

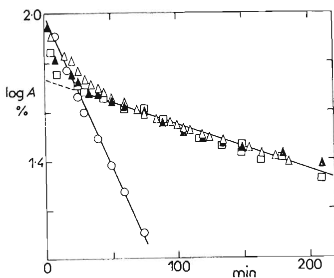


FIG. 4

Thermoinactivation of Chymotrypsin at 50°C in 0·1M-NaCl (pH 8·0)

○ Free enzyme at $[E]_0$ $1.2 \cdot 10^{-8}$ mol; product *III*: □ $1.5 \cdot 10^{-8}$ mol, ▲ $6.0 \cdot 10^{-8}$ mol, △ $1.2 \cdot 10^{-7}$ mol.

It has been shown that at low enzyme concentrations ($1{\cdot}2 \cdot 10^{-7}$M and less) no autolysis is involved in the chymotrypsin thermoinactivation process.[14]. In accordance with that, it can be seen in Fig. 4 that at the enzyme concentration $2 \cdot 10^{-8}$M the thermoinactivation of native chymotrypsin obeys the first-order kinetics. The observed thermoinactivation curve for product *III* is more complicated, probably due to the presence of several forms in the preparation that inactivate at different rates. On the other hand, the thermoinactivation kinetics of the product *III* does not depend on the starting concentration of the enzyme ($1{\cdot}2 \cdot 10^{-8} - 6 \cdot 10^{-8}$M), which means that no aggregation or autolysis is involved. This was confirmed also by the following experiment: after three hours of incubation at 50°C the reaction mixture of thermoinactivation was rapidly cooled down and kept overnight at 4°C. After rapid heating of the mixture to 50°C the thermoinactivation proceed with the same kinetics as without breakage. From the slopes of the approximated straight lines in Fig. 4 the thermoinactivation rate constants (k_{inact}) are $2{\cdot}9 \cdot 10^{-2}$ min^{-1} for free enzyme and $4{\cdot}6 \cdot 10^{-3}$ min^{-1} for product(*III*) which shows 6·3-fold stabilization of the polymer bound enzyme.

Interaction with Inhibitors

In the interaction with reversible low-molecular inhibitors indole and proflavine the polymer bound chymotrypsin behaved like the native enzyme (Table I). Significant differences became evident in the reaction with soybean trypsin inhibitor. The enzyme–inhibitor complex dissociation constant, K_i, for product *I* was 5 times and for product *III* 30 times larger than that of free chymotrypsin. These data point to a shielding effect of polymer carrier in the interaction of the polymer bound enzyme with other proteins (specific protein inhibitors, proteolytic enzymes *etc.*). Similar shielding effect of the polymer carrier was observed in the interaction of the immobilized chymotrypsin with synthetic polymeric substrates[15].

REFERENCES

1. Marshall J. J., Rabinowitz M. L.: Arch. Biochem. Biophys. *167*, 777 (1975).
2. Marshall J. J., Rabinowitz M. L.: J. Biol. Chem. *251*, 1081 (1976).
3. Westman T. L.: Biochem. Biophys. Res. Comm. *35*, 313 (1960).
4. Torchilin V. P., Reizer I. L., Smirnov V. N., Chazov E. I.: Bioorgan. Khim. *2*, 1252 (1976).
5. Svensson B.: Biochim. Biophys. Acta *429*, 954 (1976).
6. Knowles J. R., Preston J. N.: Biochim. Biophys. Acta *151*, 290 (1968).
7. Berezin I. V., Vill H., Martínek K., Yachimirskii A. K., Khludova M. S.: Mol. Biol. *1*, 843 (1967).
8. Kopeček J., Bažilová H.: Eur. Polym. J. *9*, 7 (1973).
9. Chytrý V., Vrána A., Kopeček J.: Makromol. Chem. *179*, 329 (1978).
10. Fields R.: Biochem. J. *124*, 579 (1971).
11. Sikk P. F., Paris J. P., Aaviksaar A. A.: Org. Reactivity *10*, 837 (1973).

12. Berezin I. V., Kazanskaya N. F., Klyosov A. A.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *15*, 121 (1971).
13. Antonov V. K., Vorontyntzeva T. I., Kogan G. A.: Mol. Biol. *4*, 240 (1970).
14. Martinek K., Goldmacher V. S., Klibanov A. M., Berezin I. V.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *51*, 152 (1975).
15. Kopeček J., Rejmanová P., Chytrý V.: Makromol. Chem. *182*, 799 (1981).

Translated by L. Kopecká.