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COMPARATIVE STUDY OF NATIVE AND CHEMICALLY MODIFIED CHYMOTRYPSIN AS MONOMERS, SOLUBLE POLYMERS AND MEMBRANES

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SUMMARY

α-Chymotrypsin (E.C. 3.4.21.1.) has been immobilized in the form of monomers, soluble polymers and membranes with glutaraldehyde. Chemically modified monomers (CMMs) of α-chymotrypsin were separated chromatographically on Sephadex G-100 to verify that they had the same molecular weight as the native enzyme. In addition, electrophoretic separation was carried out to determine the modifications due to the chemical reaction between free amine fractions and glutaraldehyde. The Michaelis constant of the native enzyme, CMMs, the soluble polymers and membranes was measured. It is identical for the native enzyme and CMMs, and increases with the degree of polymerization for soluble polymers and membranes. Increasing stability was observed in the following order: native enzyme, CMMs, soluble polymers and the membrane at 50°C. These experiments permitted the study of the influence of chemical modifications on the enzyme kinetics.

INTRODUCTION

When an enzyme is immobilized on a support the relevant kinetic parameters may often differ substantially from those of the free enzyme[1]. This is considered to be caused by two factors. One is the modulation of the enzyme itself through modifications of amino acid residues involved at the active or regulatory sites, and modification of the flexibility of the protein. In the other, the carrier results in diffusional limitations. After immobilization, it is difficult to separate the impact of each factor.

The changes in kinetics after insolubilization without a chemical step have previously been reported[2,3]. The purpose of the present work is to study the chemical step by measuring several kinetic constants of enzymes cross-linked with glutaraldehyde as soluble oligomers.

Little attention has been paid to enzymes immobilized in a soluble form. Polyelectrolytes bearing α-chymotrypsin have been produced in order to study the microenvironment effect[3]. The activity of dimers of bovine pancreatic ribonuclease A has been measured and compared to that of the native enzyme[5]. Protein–enzyme conjugation has been carried out for immunological techniques[6].

The modification of the Michaelis constant, K_M, due to the carrier itself has been studied by Axen *et al.*[7]. The apparent K_M has been measured for acetyl-L-tyrosine ethyl ester when α-chymotrypsin was immobilized by CNBr-activated Sephadex. Its value was ten times higher than that of the native enzyme. After enzymatic solubilization of Sephadex the new value of K_M was found to be close to that of the native enzyme.

In the present work, by varying the glutaraldehyde concentration, α-chymotrypsin was used to produce various types of α-chymotrypsin oligomers from simple chemically modified monomers to high-molecular-weight polymers[8]. The Michaelis constant and the ability to withstand high temperatures were measured for the different forms of the enzyme. It is possible to compare, under well defined conditions, native enzyme, enzyme which has reacted with the bifunctional reagent and enzyme within a proteic environment such as polymers or membrane.

MATERIALS AND METHODS

Enzyme polymer production

Soluble polymeric complexes of bovine pancreas α-chymotrypsin (E.C. 3.4.21.1.) were produced according to a previously described method[9]. To a solution (0.2 ml) containing 40 mg ml^{-1} α-chymotrypsin in 0.02 *M* phosphate buffer, pH 6.8, were added two different volumes of glutaraldehyde to give final concentrations of 0.15 and 0.03% (w/v). The solutions were kept for 30 min at 20°C. An excess of glycine was introduced to stop the reaction. The mixtures were dialysed overnight against the buffer solution.

Enzyme membrane production

The method has previously been described by Thomas *et al.*[10]. A 1-ml solution of 40 mg ml^{-1} α-chymotrypsin and 0.75% (w/v) glutaraldehyde in 0.02 *M* phosphate buffer, pH 6.8, was prepared. After polymerization a thin 4 × 4 cm membrane was obtained.

Chromatographic separation

The molecular weight of the enzyme polymer was determined using a Sephadex G-100 column. The protein elution was followed at 280 nm. The sample volume was 1 ml, the fraction size 5 ml and the flow-rate 30 ml h^{-1}.

Electrophoresis

The electrophoresis was performed using Sebia equipment. The samples, 50 μl of protein (40 mg ml^{-1}), were deposited on Cellogel plates and were run at 200 V for 30 min. Proteins were precipitated and the bands stained with a solution of 0.5% ponceau red. Protein mobilities were measured with a densitometer.

Enzyme activity measurement

Free and immobilized enzyme were tested in a $2 \cdot 10^{-3}$ *M* solution of glutaryl-L-phenylalanine-4-nitroanilide (GPNA) in 0.05 *M* Tris–HCl buffer, pH 8, in presence of 0.02 *M* $CaCl_2$. The variation in the amount of nitroanilide was followed at 410 nm. The spectrophotometer was thermostatted at 25°C.

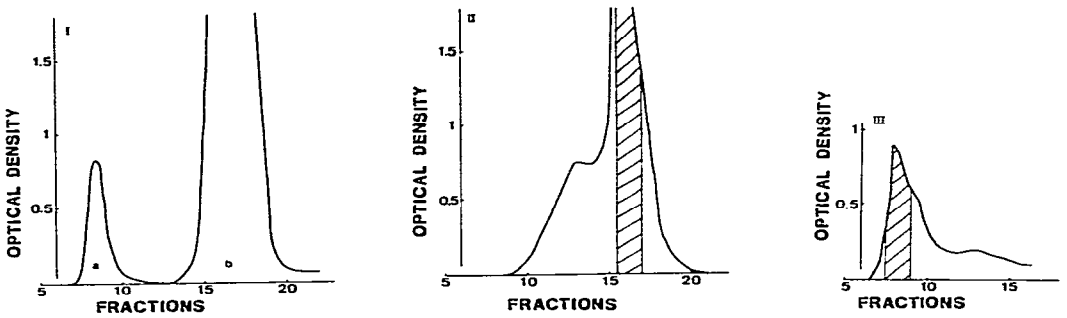


Fig. 1. Sephadex G-100 chromatography. I, Calibration of the column with blue dextran (a) and native α-chymotrypsin (b); II, chemically modified monomers; III, soluble polymers. The protein elution was followed at 280 nm. The hatched fractions were pooled and used for the kinetic studies.

RESULTS AND DISCUSSION

A solution of enzyme, placed in contact for 30 min with 0.03 % glutaraldehyde, was studied by gel chromatography (Fig. 1). It is important to note that the main peak corresponds to the elution volume of the native enzyme, and the molecules responsible for it exhibit an apparent molecular weight similar to that of the free enzyme. It is of interest to determine whether the enzyme molecules pooled from the main peak a chemically modified or not. A comparative study of the electrophoretic mobilities of both the treated and native enzyme molecules was performed. The observed profiles (Fig. 2) were significantly different. From chromatography and electrophoresis experiments, there is strong evidence for the existence of a chemically modified monomer (CMM) of α-chymotrypsin after glutaraldehyde treatment under the conditions described above.

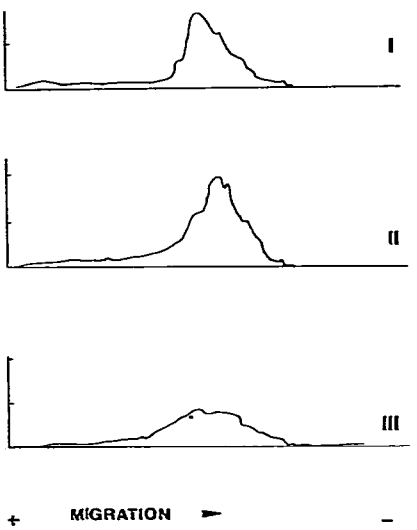


Fig. 2. Densitometer tracing of glutaraldehyde-modified α-chymotrypsin: I, native enzyme; II, chemically modified monomers; III, soluble polymers.

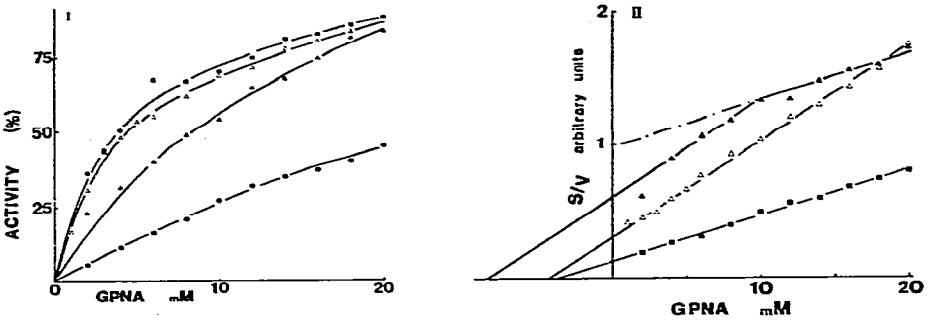
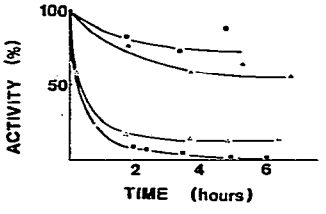


Fig. 3. I, α-Chymotrypsin activity as a function of GPNA concentration; ■, native form; ▲, CMMs; △, soluble polymers and ●, membrane. II, The ratio of substrate concentration to enzyme reaction velocity as a function of the substrate concentration.

When enzyme molecules were treated with 0.15% glutaraldehyde a considerable modification of the chromatography elution profile was observed (Fig. 1). The major part of the enzyme activity is linked to polymers excluded from the gel (molecular weight higher than 150,000). The excluded polymers were subjected to electrophoresis (Fig. 2). When compared to the above two cases, modifications of both electrophoretic mobility and profile were observed. Due to the chemical reaction between free amine fractions and glutaraldehyde molecules, the net positive charge of the enzyme at pH 4 was slightly decreased.

For the measurement of the Michaelis constant, the α-chymotrypsin kinetics were likened to Michaelian kinetics. The results obtained with native α-chymotrypsin, CMMs, polymers and membranes are given in Fig. 3. α-Chymotrypsin and CMMs exhibit similar K_M values of $3.8 \cdot 10^{-4}$ M and $4.4 \cdot 10^{-4}$ M respectively. The difference is not significant and may be within experimental error. In contrast, the apparent K_M value of polymers was found to be at least two times higher. Due to important diffusion limitations in the membrane, first order kinetics were observed for the complete range of useful substrate concentrations.

To assess the effect of cross-linking on the behaviour of α-chymotrypsin, the ability to withstand heat denaturation was also tested at 50°C for the different forms (Fig. 4). An increased thermal stability of enzymes chemically bound in an albumin



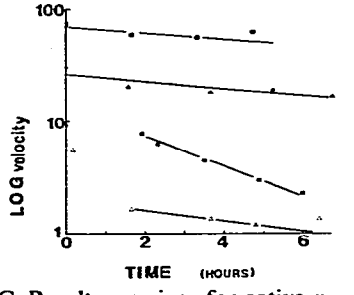


Fig. 4. Remaining enzyme activity after various incubation times at 50°C. Results are given for native α-chymotrypsin (■), CMMs (▲), soluble polymers (△) and membrane (●).

Fig. 5. Logarithmic plot of the activity against incubation times at 50°C. Symbols as in Fig. 4.

matrix has already been described, and was confirmed when the matrix was the enzyme itself. These experiments demonstrate the important rôle of the proteic environment in the enzyme stability. Increasing stability was observed in the order CMMs, polymers and membrane. Plots of log A against time (Fig. 5) gave parallel lines for monomers, polymers and membrane, and show the difference between chemically modified monomers and native enzyme molecules. Thermal stability due to the chemical modification of lysine has been observed by Tuengler and Pleiderer[11] and Cupo *et al.*[12].

CONCLUSIONS

According to these results the difference in behaviour between the native and immobilized α-chymotrypsin can be explained in terms of diffusional limitations: on the one hand, the native enzyme and the monomers chemically modified by the glutaraldehyde have the same Michaelis constant; on the other, the value of the apparent Michaelis constant increased with the degree of polymerization of the enzyme. The chemical modifications of the enzyme during the immobilization by the glutaraldehyde did not have any influence on the value of the kinetic constant. It is interesting to compare these results with those for hemoglobin immobilized by the same bifunctional reagent. The affinity for oxygen of native hemoglobin and of hemoglobin modified by glutaraldehyde is very different and changes little with the degree of polymerization for either the soluble polymers or the artificial membrane. The behaviour of immobilized hemoglobin is greatly dependent on the chemical modifications by glutaraldehyde and can't be interpreted in terms of the "freezing effect" or diffusional limitations[13].

A Michaelian enzyme like α-chymotrypsin will be little affected by the chemical modifications due to the bifunctional agent during the immobilization, except for a decrease of activity due to the direct action of glutaraldehyde on the catalytic site. On the other hand, the chemical treatment during the immobilization of an allosteric protein such as hemoglobin significantly modifies its behaviour. These results are not surprising if one considers that, in contrast to Michaelian proteins, the active sites of allosteric proteins are readily affected even at some distance.

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