IMMOBILIZATION OF THE ENZYME L-ASPARAGINASE FROM *E. coli* ON POLYSACCHARIDES.

II. COVALENT BINDING WITH SOLUBLE CM-CELLULOSE

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The covalent binding of L-asparaginase to soluble CM-cellulose has been carried out by the azide method, and some physicochemical properties of the preparation obtained have been studied. It has been established that the modified L-asparaginase possesses a higher heat stability than the native enzyme and also a greater resistance to proteolytic enzymes.

The immobilization of the enzyme L-asparaginase on insoluble polysaccharides, including insoluble CM-cellulose, has been reported previously [1]. In the present paper we give the results of the covalent binding of L-asparaginase to the soluble form of CM-cellulose (s-CMC).

The enzyme was coupled with the s-CMC by the azide method [2, 3].

When the modified L-asparaginase was desalted on Sephadex G-25, partial cleavage of the modified enzyme took place, since the reaction products containing protein issued in the form of a broadened peak with a "shoulder." Consequently the product obtained was separated into two fractions — s-CMC-asparaginase I and s-CMC-asparaginase II (Fig. 1).

The absence of noncovalent binding of the L-asparaginase in the s-CMC-asparaginases I and II was shown by gel chromatography through Sephadex-200 in a medium having a high ionic

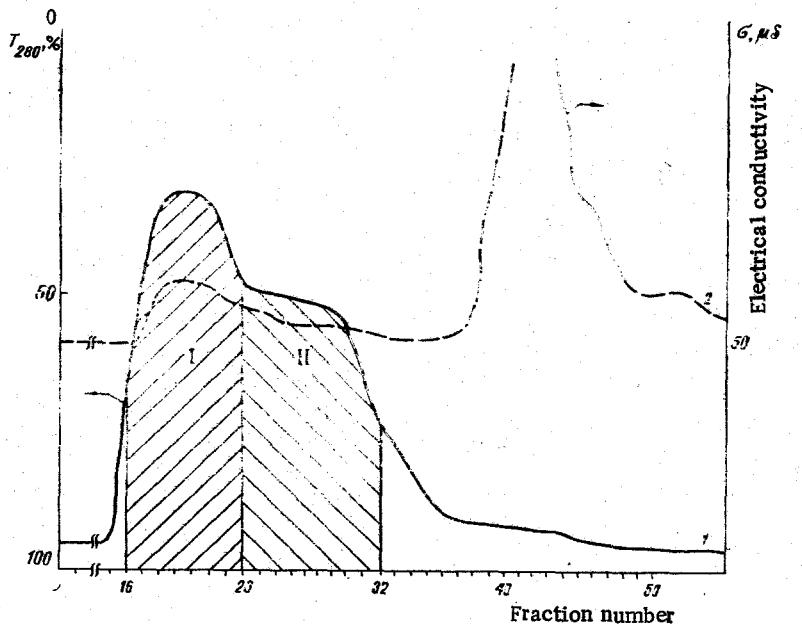

Fig. 1. Gel chromatography of the products of the reaction of the azide of CMC with L-asparaginase: I) s-CMC-asparaginase I; II) s-CMC-asparaginase II.

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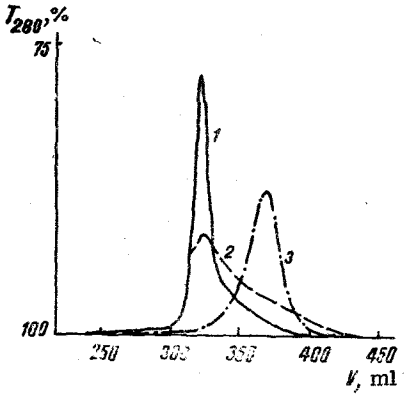

Fig. 2. Gel chromatogram through G-200 in 2 N NaCl in 0.05 M K-phosphate buffer of: 1) s-CMC-asparaginase I; 2) s-CMC-asparaginase II; 3) L-asparaginase.

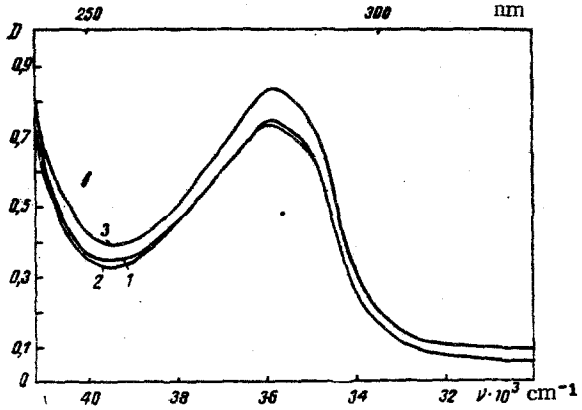

Fig. 3. UV absorption spectra of aqueous solutions of the s-CMC-asparaginase I (1), s-CMC-asparaginase II (2), and L-asparaginase (3).

strength (2 N NaCl), in which the charges of the macromolecular components are screened by the ions of the low-molecular-weight electrolyte [4] (Fig. 2), but no separation of the L-asparaginase into subunits took place (as has been shown [5], the dissociation of L-asparaginase into subunits begins in 2.5-3 N NaCl).

The activities of the freeze-dried s-CMC-asparaginase I and II are given below:

s-CMC-Asparaginase	IU/mg of Preparation	IU/mg of Protein
I	57,7	72
II	39,0	90

The UV absorption spectra in the 250-330 nm range of aqueous solutions of both s-CMC-asparaginases and of the native enzyme showed characteristic protein absorption with a maximum at 280 nm (Fig. 3). s-CMC does not absorb in this range [6].

The electrophoretic positions of the main enzyme bands of the native asparaginase and the s-CMC-asparaginases on disk electrophoresis in polyamide gel coincided, although the zones of the modified forms had a more diffuse nature (Fig. 4).

The pH optimum of the activity of the homogeneous s-CMC-asparaginase I is in the region of the pH optimum of the native enzyme, but at the same time a sharper peak of the activity curve somewhat displaced in the direction of alkaline pH values is observed (Fig. 5).

The s-CMC-asparaginase I also possesses greater heat stability than the native enzyme.

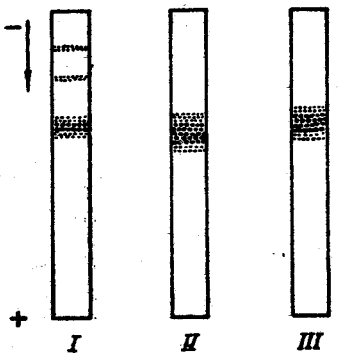

Fig. 4

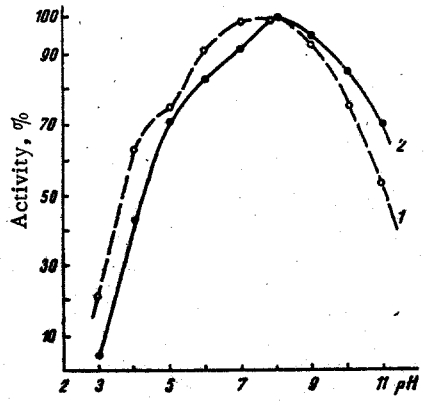

Fig. 5

Fig. 4. Electrophoresis in polyacrylamide gel at pH 8.5 of: I) L-asparaginase; II) s-CMC-asparaginase I; III) s-CMC-asparaginase.

Fig. 5. Dependence of the activity of native L-asparaginase (1) and of s-CMC-asparaginase I (2) on the pH of the medium.

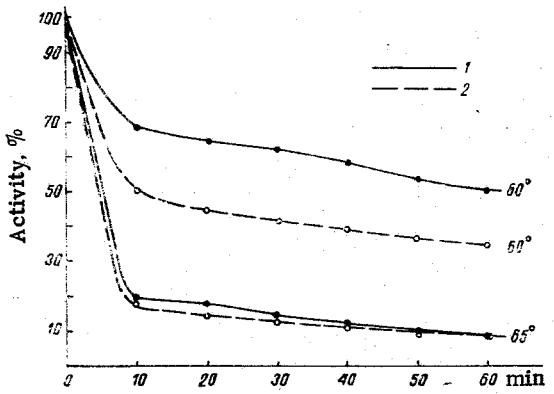

Fig. 6. Heat stability of native L-asparaginase (1) and of a s-CMC-asparaginase I (2).

While at 65°C the action of the temperature is the same as for the two forms of L-asparaginase, at 60°C the stabilizing significance of immobilization is shown (Fig. 6).

The action of proteolytic enzymes (papain, trypsin, pronase) in fairly high concentrations on the native and the s-CMC-bound L-asparaginases showed an increased resistance of the latter to proteolysis (Fig. 7). The stabilizing value of the binding of L-asparaginase to s-CMC appeared most clearly with an increase in the time of proteolysis which is an important factor in the creation of new medicinal forms of the enzyme.

EXPERIMENTAL

The work was carried out with the highly purified enzyme — *E. coli* L-asparaginase — from the Riga medical preparations factory of the Institute of Organic Synthesis of the Academy of Sciences of the Latvian SSR having a specific activity of 180 IU/mg. The soluble CMC had a degree of substitution of 0.85 and a degree of polymerization of 73.6, which corresponds to a molecular weight of 16,930.

The activities of the native and bound L-asparaginases were determined by direct Nesslerization [7], and the protein content by Lowry's method [8].

For the gel chromatography of aqueous solutions (1 ml each) of s-CMC-asparaginases I and II (20-30 mg each) we used a column of Sephadex G-100 (25×550 mm, LKB). Elution was performed with 2 N NaCl in 0.05 M K-phosphate buffer (pH 8.0), v = 0.27 ml/min.

The spectrophotometric investigation was performed on a Specord UV spectrophotometer.

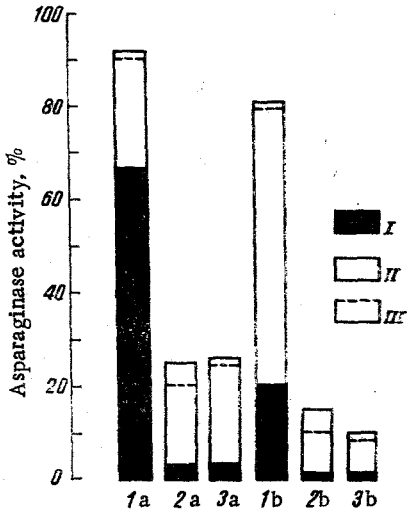

Fig. 7. Inhibition of the activity of native L-asparaginase (I), of CMC-asparaginase I (II), and of CMC-asparaginase II (III) (50 IU/ml each) at 37°C by proteolytic enzymes: 1) papain; 2) trypsin; 3) pronase; a) proteolysis for 2 min; b) proteolysis for 5 min.

Disk electrophoresis in polyacrylamide gel was performed by Davis's method [9], and the L-asparaginase activities were determined by staining in the gel according to a method which we have suggested previously [10].

Proteolysis of the solutions of native and s-CMC-asparaginases (50 IU/ml) was performed at 37°C for 2 and 5 min in K phosphate buffer (pH 8.0) with Merck papain (50 μg/ml), Spofa trypsin (100 μg/ml), and Calbiochem pronase (50 μg/ml).

The s-CMC-asparaginase was stored in the freeze-dried form.

Binding of L-Asparaginase to CMC. The CMC-asparaginase was obtained by the azide method [2, 3]. CMC in the H form (5 g) was suspended in absolute methanol and was treated with an excess of an ethereal solution of diazomethane, after which the mixture was left overnight. The precipitate was washed on a glass filter with methanol and with ether. Yield 4.8 g.

The methyl ester of CMC (4.8 g) was dissolved in 250 ml of hydrazine hydrate and the solution was heated at 39°C for 3 days. The residue after evaporation under reduced pressure was suspended in water and freeze-dried. The yield of CMC hydrazide was 4.7 g. The nitrogen content was 7.40% and the methoxy group content 1.97%.

CMC hydrazide (1 g) was suspended in 200 ml of 2% HCl and the suspension was heated at 60-70°C for 1 h for swelling and partial dissolution. Then, 26 ml of 5% $NaNO_2$ was added dropwise with stirring to the resulting suspension cooled to 0-4°C. After 45 min, the insoluble CMC azide was separated off by centrifuging with cooling at 20,000 rpm for 20 min. The water-soluble CMC azide was precipitated by pouring the supernatant into a cooled mixture of 200 ml of acetone and 70 ml of ether and was separated by centrifuging at 20,000 rpm for 30 min, and it was then washed on the filter with cooled ether and was used without delay. Yield 480 mg. It was shown viscosimetrically that the water-soluble CMC azide had a degree of polymerization of 49.9, which corresponds to M_V = 11,400.

The water-soluble CMC azide (480 mg) was dissolved in 80 ml of cooled 0.05 M borate buffer, pH 8.7, containing 440 mg of L-asparagine. The solution was left overnight at 0-4°C with stirring, the pH being maintained at 8.5-8.7 with the aid of 0.1 N NaOH. The solution was desalted on a column of Sephadex G-25 (column 30×1100 mm; eluent water; fraction volume 12 ml). The issuing broadened peak of the protein component was separated into two fractions.

SUMMARY

L-Asparaginase immobilized on soluble CM-cellulose has been obtained, and some of its physicochemical properties have been studied.

It has been established that L-asparaginase bound to soluble CM-cellulose possesses a greater heat stability and a greater resistance to proteolytic enzymes than the native L-asparaginase.

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A STUDY OF THE STRUCTURE OF THE DIOXANE LIGNINS OF THE COTTON PLANT OF VARIETY 108-F BY PROTON MAGNETIC RESONANCE

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Analysis of the PMR spectra of acetylated dioxane lignins of the cotton plant collected in various vegetation periods have shown that the DLAs have different degrees of substitution of the C_3 side chain and different degrees of condensation through the aromatic nuclei. With an increase in the time of extraction of the dioxane lignins from the plant, condensation of the lignins isolated takes place. It has been shown that in the DLAs studied the amounts of α-alcoholic hydroxyls are different.

At the present time, in spite of considerable experimental and methodological difficulties [1], the method of high-resolution proton magnetic resonance (PMR) is being used successfully in the study of various lignin preparations.

We have studied the PMR spectra of the dioxane lignins of the stems of the cotton plant of variety 108-F collected in the early period of vegetation (DLA-I) and in the flowering stage (DLA-II), of the ripe stems of the later vegetation period (F-I), and of ripe bolls (DLA-K), and also the dioxane lignins of ripe stems of the cotton plant obtained with different times of extraction (F-II, 2 h; F-III, 3 h). The isolation and characterization of the dioxane lignins has been described in previous papers [2-4]. We have recorded the spectra of the acetylated lignins since they give more valuable information.

In order to assign the chemical shifts of the protons in the PMR spectra of the dioxane lignins a correlation has been made of them with literature information [5-7]. For this pur-

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