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

V. PREPARATION AND PROPERTIES OF POLYMERIC CONJUGATES BASED ON WATER-SOLUBLE CM-CELLULOSE DIFFERING BY THE QUANTITATIVE AMOUNTS OF POLYMER

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UDC 577.155.3:547.458

A precedure has been developed for the chemical binding of L-asparaginase to soluble CM-cellulose which permits final immobilization products with different degrees of modification to be obtained. Some physicochemical and immunological properties of the samples obtained have been studied.

We have previously reported on the covalent binding of L-asparaginase with a water-soluble form of CM-cellulose (CMC) [1]. However, questions relating to the nature and degree of modification of the enzyme were not considered. The desirability of a study of these questions was dictated by the fact that in a number of cases after the lyophilization of the immobilization products their solubility in an aqueous medium deteriorated. Such a phenomenon was observed, for example, on the immobilization of catalase on water-soluble dextran [2]. One of the reasons for the deterioration in the solubility of the immobilized forms of an enzyme after lyophilization and during storage may be the fact that reactive functional groups remain in the polysaccharide support forming a component of the polymer–protein conjugate isolated. As a consequence of this, the process of forming intermolecular bonds between the protein and the polymer probably continues, which leads to the appearance of sparingly soluble or insoluble products.

The aim of the present work was an improvement in the procedure for synthesizing CMC-asparaginases permitting the regulation of the amount of bound polymer in the final immobilization product with a fixed degree of modification of the enzyme. In the final account, such investigations are necessary for studying the influence of the latter on the enzymatic, physicochemical, and biological properties of the polymeric derivatives of L-asparaginase. And this, in its turn, predetermines the choice of the best methodological variant of the immobilization of enzyme.

For the immobilization of the L-asparaginase on a water-soluble CMC we used the azide method which, as shown in [1], permits the covalent modification of the enzyme to be performed under relatively mild conditions. However, on the preparative level a defect of the method described is that the yield of water-soluble fraction of CMC azide does not exceed 50%. It was therefore of interest in the first place to obtain a completely soluble CMC-hydrazide. It could have been assumed that it was possible to achieve this by a more far-reaching modification of the initial CMC. In actual fact, after extremely prolonged treatment of the methyl ester of a CMC with the maximum theoretical possible number of ester groups with hydrazine hydride, a water-soluble CMC derivative was isolated on which about 65% of the initial carboxy groups had been converted into hydrazide groups. In order to block the remaining reactive groups of the polymer and, consequently, to stop the process of binding L-asparaginase before the desalting and lyophilization of the final immobilization products, glycine was also added to the reaction mixture. To obtain polymeric conjugates of L-asparaginase with a definite CMC content we used various weight ratios of polymer and enzyme. The results of the immobilization of L-asparaginase are generalized in Table 1.

Covalent binding with CMC azide most probably basically involved the primary ε-amino groups of the lysine residues of the enzyme, since it has been shown that in L-asparaginase

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 0009-3130/87/2304-0488$12.50

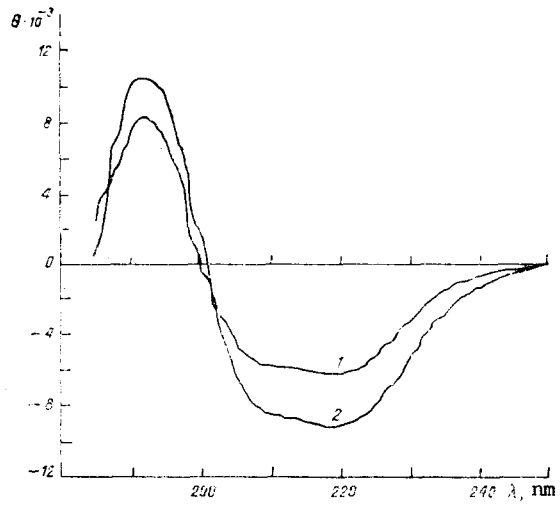

Fig. 1. CD spectra of L-asparaginase (1) and of CMC-asparaginase 2 (2).

TABLE 1. Characteristics of the Lyophilized Samples of CMC-Asparaginases

Sample of CMC-asparaginase	Amt. protein, mg/mg of preparation	CMC-asparaginase ratio, mg/mg	Activity*		Number of modified amino groups per subunit
			IU/mg of preparation	IU/mg of protein	
1	0,594	0,5/1	67,8	114,1	0,61 (2,5%)
2	0,454	1/1	41,0	90,2	5,2 (21,1)
3	0,380	2/1	16,5	43,6	9,5 (38,6%)
4	0,186	4/1	7,5	40,1	10,7 (44,6%)

*The activity of the initial L-asparaginase was 121.4 IU/mg of protein.

trinitrophenylated to the maximum degree of N-terminal amino acid leucine remains unmodified [3]. In spite of the fact that literature information on the dependence of the catalytic activity of L-asparaginase on the depth of modification of the ε-amino groups of its lysine residues is sometimes contradictory (for example, [4] and [5]), in the majority of publications it has been shown that the enzymatic activity of L-asparaginase is retained even at extremely high degrees of modification of these groups [5, 6].

Quantitative analysis of the free primary amino groups in the CMC-asparaginasses synthesized showed that with a change in the weight ratio of the reactants in the selected interval the number of lysine residues of the enzyme bound to the CMC, calculated to a subunit, increased in proportion to the amount of bound polymer. At the same time, the specific enzymatic activity decreased correspondingly. Although it was possible to compare the influence of low- and high-molecular-weight compounds of similar depths of modification with respect to the catalytic activity of the enzymes only to a certain approximation, nevertheless a comparison of the result obtained with earlier results [7] in which the influence of the depths of trinitrophenylation of L-asparaginase on the activity of the enzyme appears to be of definite interest. The authors concerned showed, in particular, that as a result of the trinitrophenylation of two or three NH_2 groups of L-asparaginase 26% of its activity was lost, while the modification of eight NH_2 groups led to the loss of 68% of its enzymatic activity. In the case of the modification of L-asparaginase with CMC azide, however, losses of 26 and 67% of enzymatic activity took place on the modification of five and 10-11 NH_2 groups, respectively, i.e., the "polymer" stabilizing effect of the polyfunctional high-molecular-weight modifying agent — CMC azide — was shown at different depths of modification of this subunit protein. It is important to note that in all cases a reproducibility of the final degree of modification of the L-asparaginase was achieved and the CMC-asparaginases obtained in this way possessed good solubility in aqueous media.

According to the results of CD-spectroscopy (Fig. 1), the covalent linkage by the azide method by the L-asparaginase with CMC macromolecules does not introduce substantial changes into the conformation of the enzyme.

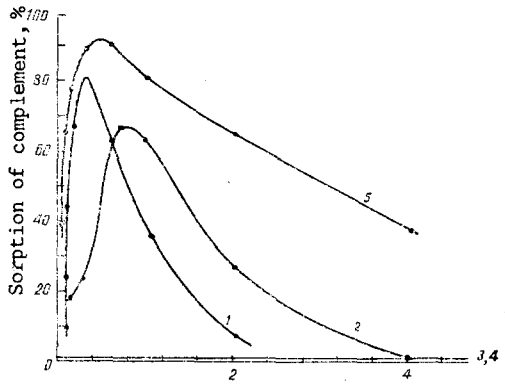

Fig. 2. Binding of complement with CMC-asparaginases 1, 2, 3, and 4 (1-4) and of L-asparaginase (5) in the presence of antibodies to native L-asparaginase.

TABLE 2. Results of Isoelectric Focusing in Polyacrylamide Gel

pH interval	Number of protein components	
	L-asparaginase	CMC-asparaginase 2
3,0—4,0		4
4,0—4,5	5	6 (4,2; 4,25; 4,6)*
4,5-5,0	6 (4,4; 4,5; 4,8)*	3
5,0-6,0	—	—
6,0—9,5	—	—

*The pH values corresponding to the IEPs of the main protein components.

An investigation of the component composition of CMC-asparaginase 2 by isoelectric focusing in polyacrylamide gel showed that the main protein zones of the preparation were characterized by lower values of the isoelectric points (IEPs) than the corresponding zones of the unmodified L-asparaginase (Table 2). At the same time, as was expected, the CMC-asparaginase was more heterogeneous than the L-asparaginase and contained about 13 protein zones with different IEPs.

However, in a study of the antigenic composition by immunoelectrophoresis it was found that the native enzyme and the samples of CMC-asparaginases each contained a single antigen. Immunoelectrophoresis also showed that L-asparaginase and CMC-asparaginases differed substantially in their electrophoretic mobilities, while the electrophoretic mobilities of the modified form of L-asparaginase obtained at various ratios of CMC and enzyme differed only slightly:

Sample	Electrophoretic mobility $cm^2 \times V^{-1} \times sec^{-1}$
L-Asparaginase	-7×10^{-4}
CMC-Asparaginase 2	-13.6×10^{-4}
CMC-Asparaginase 3	-14.4×10^{-4}

An investigation of the capacity of the CMC-asparaginases for interacting with antibodies to the native L-asparaginase (Fig. 2), showed that a decrease in antigenic affinity with the native enzyme was characteristic for all the samples synthesized, and even a complete absence of the above-mentioned introduction of samples of CMC-asparaginase 3 and 4 containing a fairly large amount of bound polymer. This means that there is a possibility of

obtaining an enzyme preparation with a qualitatively changed antigenicity which, in its turn, is an important factor in the creation of new L-asparaginase preparations lacking cross-antigenicity [8]. A subsequent study of the antileukemia properties of the samples will permit us to find the most effective immobilized form of L-asperaginase.

EXPERIMENTAL

Highly purified E. coli L-asparaginase (Institute of Organic Synthesis of the Latvian SSR Academy of Sciences) with a specific activity of 120-150 IU/mg of protein was used. The soluble CMC-Na (Namangan chemical factory) had a degree of substitution (DS) of 0.85. The degree of substitution of the CMC was determined by the precipitation of its uranyl salt [9].

The activities of the native and the immobilized L-asparaginases were determined by the method of direct Nesslerization [10] and the protein contents by Lowry's method [11]. The numbers of free primary amino groups in the CMC-asparaginases were determined as described in [7].

CD spectra were recorded on a Jobin Yvon Mark III dichrograph (France). The dichrograph was calibrated with respect to the standards 10-camphorsulfonic and epiandrosterone. To record the spectra use was made of cells of fused quartz (Hellma) with optical pathlengths of 1 cm in the spectral interval of 300-250 nm and of 0.1-0.01 cm in the interval of 250-185 nm. The concentration of the solution was ~10^{-3} M calculated to the protein content. The slit program corresponded to 20 Å. The spectra obtained were treated on a WANG 2200 VP computer (USA) with devices for the input of graphical information with the aid of a specially developed packet of programs [12]. Molecular ellipticities, θ, were measured in deg·cm^2/dmole.

The antigenic compositions and electrophoretic mobilities of the CMC-asparaginases were studied by immunoelectrophoresis [13] on glass plates in 1% agarose gel (Sigma) on a Multiphor instrument (LKB) in barbital sodium buffer, pH 8.2. Solutions containing 10 mg of protein/ml were deposited in wells with a diameter of 2.5 mm. The time of electrophoresis was 1.5 h at a current of 50 mA.

Immunoprecipitation was performed with hyperimmune rabbit serum to native L-asparaginase interacting with the test antigen in the indirect hemagglutination reaction in a dilution of 1:10,000. Electrophoretic mobilities (U) were calculated from the formula

$$U = D/H \cdot t,$$

where D is the distance travelled by the protein, cm; t is the time of electrophoresis, sec; and H is the electric field strength, V/cm;

$$H = U_{end}/\ell,$$

where U_{end} is the voltage at the ends of the plate, V; and ℓ is the length of the plate, cm.

The quantitative complement fixation test, CFT, was carried out as described in [14]. The results were evaluated from the efficiency of the sorption of the complement by the antigen–antibody complex. The amount of hemoglobin liberated as the result of the hemolysis of erythrocytes was measured spectrophotometrically on an ABA-100 bichromatic analyzer (USA).

Synthesis of the Methyl Ester of CMC. A solution of 5.5 g of CMC-Na in 60 ml of H_2O was brought to pH 2.0 by the addition of 0.1 N HCl. The CMC in the H form was precipitated by the dropwise addition of 200 ml of ethanol to the CMC solution and was isolated by centrifugation at 6000 rpm for 30 min and was then washed on the filter with ethanol followed by methanol. The free CMC-H isolated in this way was suspended in 100 ml of methanol and was treated with an ethereal alcoholic solution containing an eightfold excess (calculated on the carboxymethyl groups of the CMC) of diazomethane. The reaction mixture was left at room temperature for 24 h. The bulk of the product was separated off by filtration, and the low-molecular-weight fractions, after evaporation of the filtrate to a volume of 5-10 ml, were precipitated with acetone. The fractions were combined and were washed on the filter with acetone. Yield 4.6 g. Found, %: OCH_3 11.2. Calculated: DS (with respect to the groups of the carboxymethyl methyl ester) 0.85.

Synthesis of a Water-Soluble CMC Hydrazide. A solution of 4.6 g of CMC-OCH_3 in 200 ml of hydrazine hydrate was kept at 38°C for 6 days. Then it was filtered and was concentrated by evaporation under reduced pressure to a syrupy consistency and was poured dropwise into

ethanol. The further purification of the CMC hydrazide that precipitated was carried out by its reprecipitation four times from aqueous solution into ethanol. Then it was redissolved in water and the solution was freeze-dried. Yield 4.4 g. Found, %: OCH_3 3.41; N 6.32. Calculated: DS (with respect to carboxymethyl ester groups) 0.30; DS (with respect to carboxymethyl hydrazide groups) 0.55.

The synthesis of a water-soluble CMC azide was carried out as in [1].

Binding of the L-Asparaginase to the CMC Azide. The appropriate amount of CMC azide was added to 10 ml of a cooled solution of 0.05 M borate buffer, pH 9.2, containing 15 ml of L-asparaginase (weight ratios of CMC to L-asparaginase 0.5:1, 1:1, 2:1, and 4:1). After the reaction mixture had been stirred at 0-4°C for 12 h, glycine was added to it in 2 to 5-fold excess with respect to the given amount of CMC, and stirring was continued for another 2 h. The solution was deposited on a column (25 × 1000 mm, LKB) of Sephadex G-200. Elution was performed in 0.5 N NaCl in 0.05 M K phosphate buffer, pH 8.0 at the rate of 0.21 ml/min. The fractions containing CMC-asparaginase and the protein peak issuing first on the chromatogram were combined, desalted against distilled water, and lyophilized.

I. A. Vosekalna (Institute of Organic Synthesis of the Latvian SSR Academy of Sciences) participated in the recording of the CD spectra.

CONCLUSION

The method of obtaining E. coli L-asparaginase immobilized on soluble CM-cellulose has been improved with the use of the azide method of binding. Completely soluble intermediate and final products of the synthesis have been obtained.

The possibility has been shown of obtaining immobilized forms of L-asparaginase with different degrees of modification of the enzyme differing in the quantitative content of the polymer.

It has been established that enzymatic, electrophoretic, and immunological properties of CMC-asparaginases depend on the amount of CM-cellulose present in the polymeric conjugates of the enzyme.

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