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

III. COVALENT BINDING WITH 3-BROMO-2-HYDROXYPROPYLDEXTRAN

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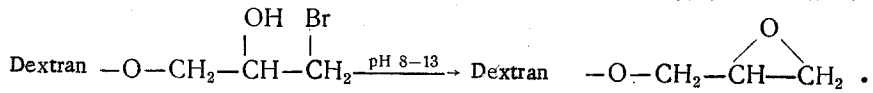
The synthesis of immobilized L-asparaginase on 3-bromo-2-hydroxypropyldextran has been effected. Some physicochemical properties of the preparations obtained have been studied. A stabilizing effect of the modification on the heat stability of the L-asparaginase has been detected. The influence of the bound polymer on the antigenic properties of the enzyme have been established.

The immobilization of L-asparaginase on some insoluble polysaccharides [1] and on soluble CM-cellulose [2] has been reported previously. In the present paper we give the results of the coupling of L-asparaginase with an activated dextran containing reactive 3-bromo-2-hydroxypropyl groups (BHP-dextran).

In an alkaline medium, BHP-dextran is readily converted into the corresponding epoxide derivative [3]:



Under these conditions, substances containing nucleophilic groups readily couple with the dextran. The binding of the L-asparaginase with the BHP-dextran was carried out at pH 10.5 where the primary amino groups of the amino acid residues may take part in the reaction preferentially, although the possibility of interaction with the hydroxy groups of tyrosine residues, as well, is not excluded. Here account was taken of the presence in the L-asparaginase, including their presence on the surface of the protein globule, of a considerable number of amino groups, particularly lysine ε-amino groups [4], and the good results of the binding of the enzyme through amino groups with other polymers [1, 2, 5].

According to the results of electrophoresis, the optimum binding of the L-asparaginase and the dextran was achieved after synthesis for 24 h at a ratio of the reactants of 1:10, respectively. Under these conditions, practically no contamination with the native enzyme was observed (Fig. 1). Judging from the increase in the electrophoretic mobility of the main enzyme zone, the molecule of the modified L-asparaginase had acquired a more negative charge, which probably took place mainly as the result of modification of the amino groups of the enzyme.

Figure 2 shows a gel chromatogram of the modified L-asparaginase obtained on the passage of the reaction mixture (weight ratio of enzyme to dextran 1:10) through Sephadex G-200. Two protein fractions containing the modified enzyme (Dn-asparaginase I and Dn-asparaginase II) were isolated and characterized; they differed in molecular weights, compositions, and activities:

Dn-asparaginase	Protein, γ/mg of preparation	IU/mg of preparation	Activity IU/mg of protein	Yield, %	Dextran, γ/mg of preparation
I	64.9	8.8	136.1	75.5	973.0
II	73.4	5.8	78.5	43.6	952.4

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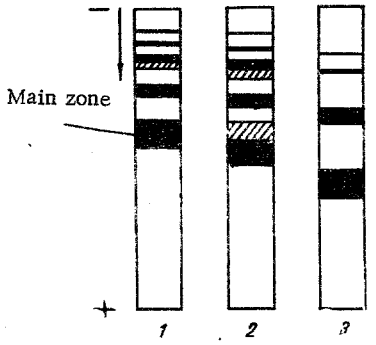

Fig. 1

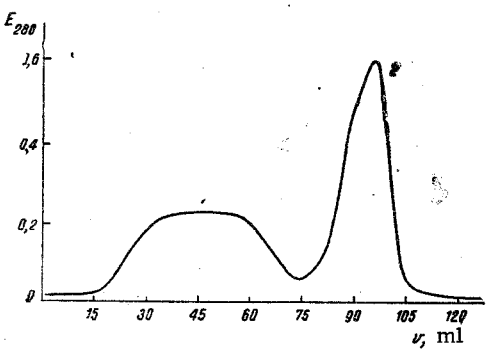

Fig. 2

Fig. 1. Electrophoresis in polyacrylamide gel: 1) L-Asparaginase; 2 and 3) reaction mixtures containing L-asparaginase and BHP-dextran in weight ratios of 1:1 and 1:10, respectively.

Fig. 2. Fractionation of the modified L-asparaginase on Sephadex G-200 (in a weight ratio of enzyme to polymer of 1:10): 1) Dn-asparaginase I; 2) Dn-asparaginase II.

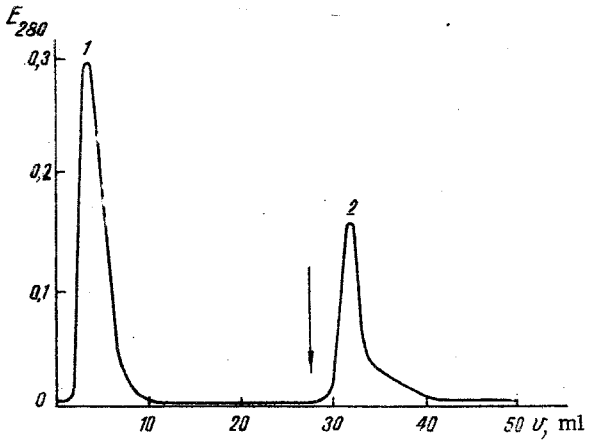

Fig. 3. Chromatographic separation on (concanavalin A)-Sepharose of the reaction mixture at a weight ratio of L-asparaginase and BHP-dextran of 1:1. The arrow shows the beginning of elution with methyl α-D-glucopyranoside.

The high-molecular-weight fraction, issuing as a broadened peak, contained a larger amount of added dextran than the low-molecular-weight fraction and possessed a higher enzymatic activity.

To elucidate the possibility of the purification and isolation of the immobilized L-asparaginase we used biospecific chromatography on (concanavalin A)-Sepharose. Under certain conditions of the medium, this sorbent specifically sorbs only branched dextran molecules, including those of enzyme–dextran conjugates [6]. In the latter case, as the result of chemical binding cross-linkages are formed in the chains. Free dextran, thanks to the linearity of its molecule, and also native enzymes, are usually not sorbed. Branched dextrans and their derivatives are readily desorbed by affinity reagents and, in particular, by methyl α-D-glucopyranoside.

In the purification of the Dn-asparaginase on the (concanavalin A)-Sepharose, the first protein fraction of the 0.05 M Tris-HCl buffer eluate in which the reaction mixture was eluted (enzyme–dextran ratio 1:1) contained the native enzyme (Fig. 3). No dextran was detected in this fraction. Subsequent elution by the same buffer containing 0.1 M methyl

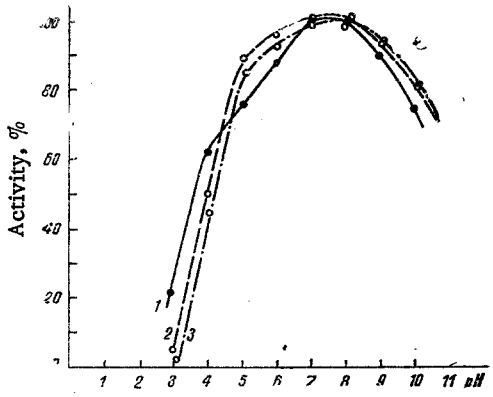

Fig. 4. Dependence of enzymatic activity on the pH of the medium: 1) L-asparaginase; 2) Dn-asparaginase I; 3) Dn-asparaginase II.

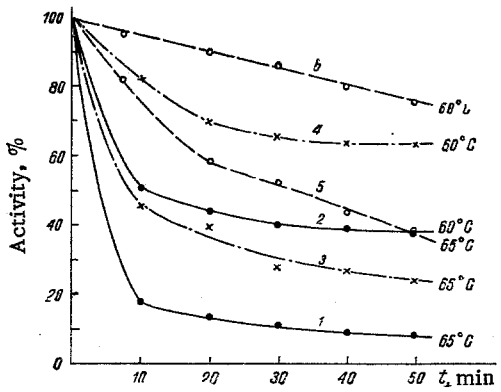

Fig. 5

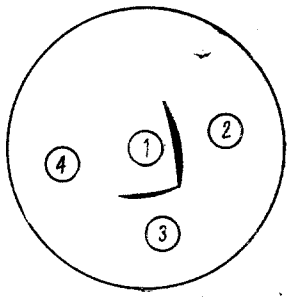

Fig. 6

Fig. 5. Thermal stability of native L-asparaginase (1, 2), Dn-asparaginase I (3, 4), and Dn-asparaginase (5, 6).

Fig. 6. Double radial immunodiffusion in agar gel: 1) antiserum to L-asparaginase; 2) L-asparaginase; 3) Dn-asparaginase II; 4) Dn-asparaginase I.

α-D-glucopyranoside provided the possibility of desorbing the Dn-asparaginase, which issued as a single peak. When a reaction mixture with a weight ratio of the reactants of 1:10 was passed through the column, no native enzyme was detected, and, as in the first case, the Dn-asparaginase issued as a single peak, with a specific activity of 102 IU/mg of protein.

Thus, biospecific chromatography on (concanavalin A)-Sepharose can be used for purifying Dn-asparaginase, which is not separated into fractions during this process, i.e., it issues in the heterogeneous state. Consequently, we studied in more detail the Dn-asparaginase I and Dn-asparaginase II isolated by gel chromatography on Sephadex G-200.

As can be seen from Fig. 4 the pH optimum of the activities of both Dn-asparaginases differed little from that of the native enzyme, which has its maximum activity at pH 7-8. At the same time, a wider plateau of the activity maximum was found, particularly in the region of acid pH values.

The coupling of L-asparaginase with BHP-dextran leads to an increase in the thermal stability of the native enzyme; however, this is inversely proportional to the amount of polymer bound, which obviously affects the conformation of the protein globule in some way (Fig. 5).

The latter hypothesis was also confirmed by the results of double radial immunodiffusion, from which it follows that antiserum containing anti-L-asparaginase does not act on

the high-molecular-weight fraction of the modified enzyme (Fig. 6). This shows a complete change in the antigenic properties of Dn-asparaginase I.

EXPERIMENTAL

Highly purified *E. coli* L-asparaginase produced by the Riga medical preparations factory of the Institute of Organic Synthesis of the Academy of Sciences of the Latvian SSR with a specific activity of 180 IU/mg of protein and BHP-activated dextran with a molecular weight of 70,000 (BHP-activated dextran 70, Sweden) were used.

The activities of the native and modified L-asparaginases were determiend by the method of direct Nesslerization [7], protein content by Lowry's method [8], and dextran content by Dubois's method [9] (dextran T 70 with a molecular weight of 70,000 (Pharmacia) was used as standard).

Disc electrophoresis in polyacrylamide gel was carried by Davis's method [10], the enzymatic activity being determined by staining [11].

The immunochemical properties of the immobilized L-asparaginase were investigated by double radial immunodiffusion in agar gel according to Ouchterlony [12]. For immunochemical analysis we used guinea pig antiserum obtained to L-asparaginase. Immunodiffusion was carried out at room temperature for 6 days, after which the proteins that had not been precipitated were washed out from the agar gel with 0.15 M NaCl solution for 12 h and were dried at room temperature and stained with Amido Black 10B.

The dependence of enzymatic activity on the pH of the medium was investigated in 0.05 M universal buffer (pH 2-12).

Binding of L-Asparaginase with BHP-Dextran. An appropriate amount of BHP-dextran was dissolved in 10 ml of 0.1 M carbonate buffer, pH 10.5, 12.6 g of L-asparaginase was added, and the mixture was stirred at room temperature for a day. Then it was purified by chromatography.

Gel chromatography was carried out in a column (25 × 1000 mm, LKB) of Sephadex G-200. Elution with water was at the rate v = 0.25 ml/min.

The affinity chromatography of the Dn-asparaginase (0.4 ml of reaction mixture containing 10 mg of protein) was performed on a column (12 × 150 mm, LKB) containing (concanavalin A)-Sepharose (Pharmacia) with elution by means of 0.05 M Tris-HCl buffer (pH 7.6) containing 1 M NaCl, and 10^{-3} M $CaCl_2$, $MnCl_2$, and $MgCl_2$ [13]. Desorption of the Dn-asparaginase was carried out in the presence of 0.1 M methyl α-D-glucopyranoside (USA) in the initial buffer. The amounts of protein and dextran were determined after ultrafiltration.

The Dn-asparaginase was stored in lyophilized form.

SUMMARY

The synthesis of L-asparaginase immobilized on 3-bromo-2-hydroxypropyldextran has been effected.

The influence of the bound polymer on some physicochemical and immunochemical properties of the preparations obtained has been established.

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DISSOCIATIVE IONIZATION OF FUSIDIC ACID AND THE PRODUCTS OF ITS THERMOLYSIS IN THE TEMPERATURE RANGE OF 30-250°C

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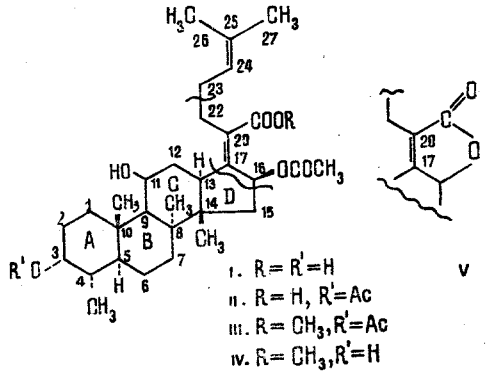
The results are reported of a study of the dissociative ionization of fusidic acid and its derivatives, and also of an investigation of the products of its thermal degradation by the mass-spectrometric method. A scheme of fragmentation of the antibiotic under the action of electron impact is suggested.

The aim of the present work was a study of the processes of fragmentation of the antibiotic fusidic acid (I) and some of its derivatives (II-V) under the action of electron impact, and an investigation of the products of its thermal degradation in the temperature range of 30-250°C.



It is known that a strict consideration of the initial processes of dissociation of the molecular ion M^+ permits a possible mechanism of the thermal destruction of the initial compound to be suggested [1-3].

Under the conditions of electron-impact mass-spectrometry, even with the low-energy (14 eV) ionization of the electrons, no M^+ peak was observed in the mass spectrum of compound (I). The M^+ peaks were also absent from the mass spectra of the 3-acetate of (I) and of its methyl ester (II, III) (Table 2). The correspondence of compounds (I-IV) to their empirical formulas was established by the method of field desorption. The purities of the substances mentioned were checked by means of this method. In contrast to fusidic acid itself, in the field-desorption mass spectra of which only the M^+ peak was recorded, for its derivatives (II-IV) the molecular weights were determined by the M^+ and $(M+1)^+$ ions. The field-desorption mass spectra of compounds (I-IV) (Table 1) are also characterized by the presence of the fragmentary ions $(M-59)^+$ and $(M-60)^+$, the formation of which is due to the elimination of the particles CH_3COO and CH_3COOH from the C_{16} position of M^+.

According to the results of high-resolution mass spectrometry, in the electron-impact mass spectrum of (I) the peak of the ion with the highest molecular weight has the empirical

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