8. V. I. Rozengart and O. E. Sherstobitov, The Selective Toxicity of Organophosphorus Insectoacaricides [in Russian], Leningrad (1978).
9. M. I. Kabachnik, A. A. Abduvakhabov, I. I. Agabekova, A. P. Brestkin, R. I. Volkova, N. N. Godovikov, E. I. Godyna, S. S. Mikhailov, M. Ya. Mikhel'son, V. I. Rozengart, E. V. Rozengart, and R. V. Sitkevich, Usp. Khim., 39, 1050 (1970).
10. G. L. Ellman, D. R. Cenntney, V. Anders, and K. M. Reather, Biochem. Pharm., 7, 88 (1961).
11. V. A. Yakovleb, The Kinetics of Enzymatic Catalysis [in Russian], Moscow (1965).
12. A. Cornish-Bowden, Principles of Enzyme Kinetics, Butterworths, London (1976).

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

IV. COVALENT BINDING WITH DIALDEHYDE-DEXTRAN

A. S. Karsakevich, S. N. Shulichenko, M. R. Buka, I. A. Vina, and A. P. Kashkin

UDC 577.155.3:547.458

The synthesis has been effected of immobilized *E. coli* L-asparaginase on medical dextran — poliglyukin. The influence of the bound polymer on some physicochemical properties of the final products have been studied. An increased resistance to heat and stability on storage of the immobilized forms of L-asparagine in comparison with a native enzyme has been found. It has been shown that the polymer modification of L-asparaginase leads to a decrease in the antigenic affinity of the immobilized enzyme as compared with the native enzyme.

The most promising among the polymers used as supports for the immobilization of enzymes for medical purposes are dextrans, which is due to their high degree of biocompatibility and their utilization in the organism [1]. We have previously reported the immobilization of the antileukemia enzyme *E. coli* L-asparaginase on 3-bromo-2-hydroxypropyldextran (Sweden) [2]. In the present paper we give the results of the immobilization of L-asparaginase on a domestic dextran — poliglyukin. For direct binding of the enzyme we used poliglyukin previously oxidized by the periodate method to dialdehyde-poliglyukin. The labile azomethine bonds formed between the enzyme and the aldehyde groups of the polymer were reduced with sodium tetrahydroborate, which also lowered the excess of aldehyde groups in the poliglyukin.

A method has been described for obtaining L-asparaginase bound to dextrans by azide and cyanogen bromide methods [3, 4] using toxic reagents, which is impermissible in the creation of a medicinal preparation for intravenous purposes such as L-asparagine. The method given in the present paper avoids the use of toxic reagents. At the same time, the activation of poliglyukin is carried out in the production of streptodekaz. In the literature, this method has been described for the immobilization of *Erwinia carotovova* L-arparaginase [5-9]. At the same time, in clinics both in the USSR and abroad only *E. coli* L-asparaginase is widely used for treating diseases. The search for effective immobilized forms of the latter based on a biocompatible polymer used in medicine therefore acquires particular importance.

In the development of the optimum conditions for the synthesis of immobilized L-asparaginase it was established that the best results with respect to completeness of binding and yield of enzymatic activity are obtained at pH 8.5 and a temperature of 20 ± 2°C in the course of 3 h of synthesis at initial weight ratios of enzyme and poliglyukin of 1:2, 1:4, 1:6, and 1:8. The characteristics of the final products are given in Table 1.

The depth of binding of L-asparaginase with poliglyukin was estimated qualitatively by column gel chromatography on Sephadex G-200 and electrophoretically. As can be seen from Fig. 1, the rate of elution of the modified forms of L-asparaginase was higher than that of the native enzyme, which indicates an increase in the molecular weights of the final products.

Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga. All-Union Scientific-Research Technological Institute of Antibiotics and Enzymes for Medical Purposes, Leningrad. A. Kirkhenshtein Institute of Microbiology, Academy of Sciences of the Latvian SSR, Riga. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 766-771, November-December, 1986. Original article submitted June 19, 1986.

 0009-3130/86/2206-0712$12.50

TABLE 1. Characteristics of Lyophilized Samples of L-Asparaginase Bound to Poliglyukin

Sample No.	Initial weight ratio of enzyme to polymer	Amount, mg/mg of the preparation		Enzymatic activity, IU/mg		Number of ε-amino groups of the enzyme bound to the poliglyukin*
		of protein	of poliglyukin	of protein	of poliglyukin	
1	1:2	0,324	0,69	77,5	25,12	7,2
2	1:4	0,231	0,80	71,5	16,30	10,5
3	1:6	0,151	0 86	70,2	10,90	14,0
4	1:8	0,134	0,89	75,3	10,10	16,8

E. coli L-asparaginase contains 96 lysine amino groups.

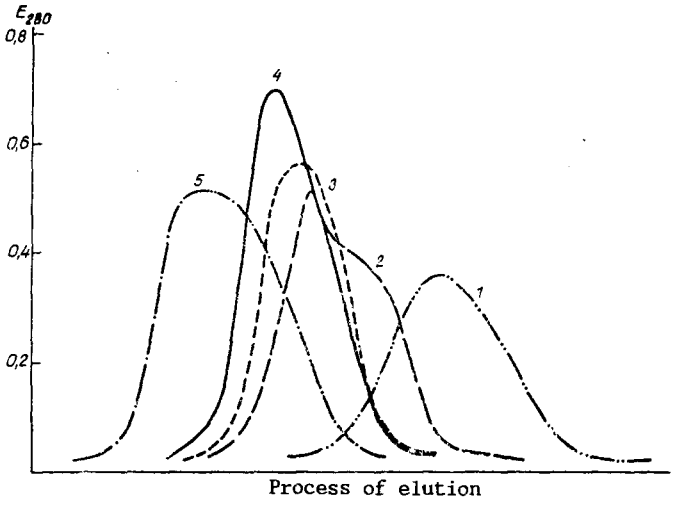

Fig. 1

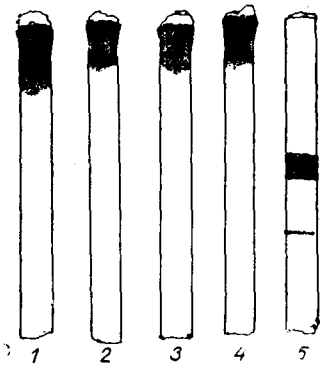

Fig .2

Fig. 1. Gel chromatography on Sephadex G-200 of native L-asparaginase (1) and of the enzyme bound to poliglyukin in weight ratios of 1:1 (2), 1:2 (3), 1:4 (4), and 1:8 (5).

Fig. 2. Electrophoresis in polyacrylamide gel: 1, 2, 3, 4) samples of immobilized L-asparaginase; 1, 2, 3, and 4, respectively (see Table 1); 5) native L-asparaginase.

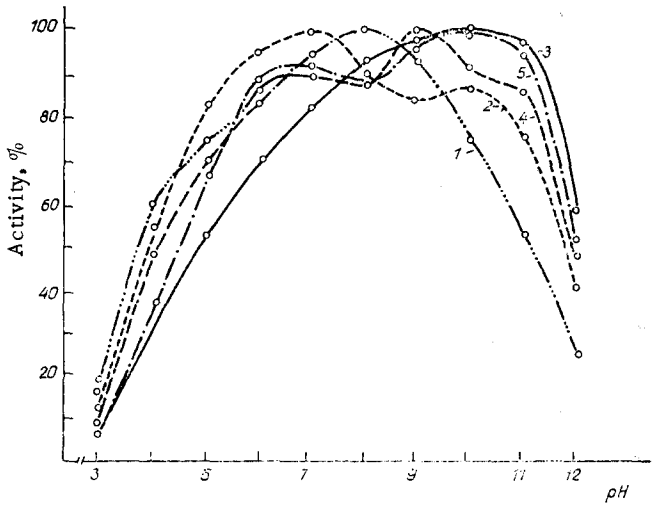

Fig. 3. Dependence of enzymatic activity on the pH of the medium: 1) L-asparaginase; 2, 3, 4, 5) samples 1, 2, 3, and 4 of immobilized L-asparaginase, respectively (see Table 1).

At a weight ratio of enzyme to polymer of 1:1, the protein peak on a chromatogram showed the presence of native L-asparaginase. Beginning at a weight ratio of 1:2, this impurity was no longer detected, which showed the complete binding of the enzyme. Similar results, but with less pronounced differences in the protein peaks recorded were obtained in gel chromatography on Toypearl-HW-55 Fine.

Electrophoresis, the results of which are presented in Fig. 2, also showed the possibility of checking the presence or absence of the native enzyme in samples of immobilized L-asparaginase. It revealed a difference between the electrophoretic behavior of the polymer conjugates and that of native L-asparaginase. This confirmed the very fact of the chemical modification of the enzyme. The immobilization products consisted of high-molecular-weight derivatives not migrating in the gel. Thus, the results of electrophoresis agreed well with those of gel chromatography. It must be mentioned that the procedure for obtaining immobilized L-asparaginases that we have developed excludes the presence of unbound poliglyukin in the final products. Small amounts of the latter, if such are present, are readily separated from the immobilization products by ultrafiltration, which is the main stage of the purification and concentration of the samples obtained.

The depth of modification of the L-asparaginase with respect to ε-amino groups of the lysine residues was evaluated by the trinitrophenylation of the native and bound enzymes (Table 1).

The figures in Table 1 lead to the conclusion of the possibility of obtaining immobilized forms of L-asparaginase with different amounts of polymer. An increase in the amount of poliglyukin bound to the enzyme and consequently, in the molecular weight of the polymer conjugate was accompanied by an increase in the depth of modification of the L-asparaginase. However, these factors had little effect on the final yield of enzymatic activity, which ranged between 55 and 60%.

An investigation of the pH dependence of the activity of the dextran conjugates of L-asparaginase (Fig. 3) showed a sharp change in the optimum state of ionization of the enzyme as a result of modification which appeared in the form of secondary activity maxima at pH 9-10. Only for sample 2 (Table 1) was some shift in the pH dependence of the catalytic activity in the direction of alkaline pH values with a broader plateau of the activity optimum observed. The appearance of the additional activity peaks shows a heterogeneous nature of the samples obtained. To some extent, this can be explained by the heterogeneity with respect to the molecular weight of the initial poliglyukin. Similar phenomena have been observed with other modifications of L-asparaginase [10] as a result of which, as in our case, it was only the mean degree of modification that was determined analytically.

The binding of L-asparaginase to poliglyukin led to a considerable stabilization of the enzyme which was shown primarily on the storage of the enzyme preparations. The results are given below:

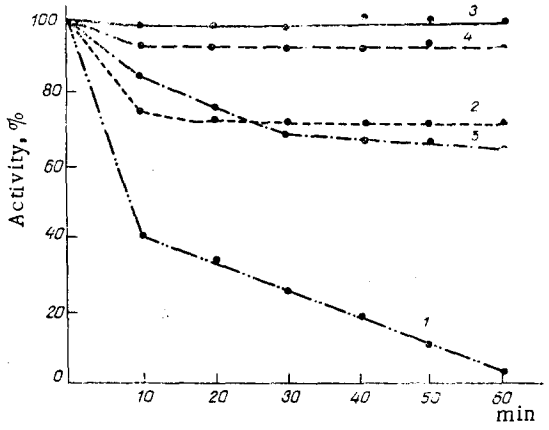

Fig. 4

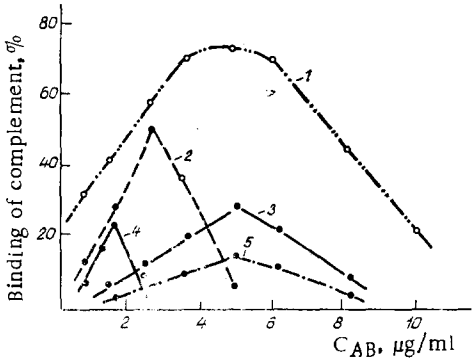

Fig. 5

Fig. 4. Thermal stability at 55°C of native L-asparaginase (1) and of immobilized L-asparaginase samples 1 (2), 2 (3), 3 (4), and 4 (5) (see Table 1).

Fig. 5. Interaction of asparaginase antibodies with native L-asparaginase (1) and with the immobilized samples 1 (2), 2 (3), 3 (4), and 4 (5) (see Table 1).

Conditions of Storage	L-asparaginase enzymatic activity, %	
	Native	Immobilized
In concentrated aqueous solution, 5°C, 6 months	0	60-70
In lyophilized form, 5°C, 2 years	20-30	90-100

The immobilization of the L-asparaginase also increased its thermal stability under conditions in which the native enzyme is rapidly inactivated (Fig. 4).

The influence of the polymer modification of the L-asparaginase can be clearly seen in a comparative study of the antigenic properties of samples of native L-asparaginase and its immobilized forms. It was found that the modification of the enzyme by poliglyukin led to a change in its antigenic activity. The capacity of the immobilized L-asparaginase for interacting with antibodies to the native enzyme decreased with an increase in the amount of polymer in the preparation, i.e., with an increase in the number of points at which it was found to the enzyme. The experimental results are shown in Fig. 5. It can be seen that the antigenic structure of the L-asparaginase underwent quantitative changes proportional to the degree of its modification. Sample 4 possessed the least antigenic affinity in relation to the native enzyme.

Thus, the modification of L-asparaginase by poliglyukin is an effective means of changing the antigenicity of the enzyme which may in future permit the use of the immobilized enzyme (if a sufficient antileukemia activity is present) in those cases of treatment of diseases where resistance to the native enzyme has developed in the organism. The latter is one of the main reasons for the fall in the therapeutic efficacy of L-asparaginase as an antileukemia drug.

EXPERIMENTAL

E. coli L-asparaginase from the Institute of Organic Synthesis of the Latvian SSR Academy of Sciences with a specific activity of 130-140 IU/mg of protein and an aqueous solution of oxidized poliglyukin (dialdehyde-dextran) with a concentration of 50 mg/ml and a degree of oxidation of 24 (number of doubly oxidized glucose rings per 100 elementary units of the dextran) were used.

Enzymatic activity was determined by the method of direct Nesslerization [11] and protein contents spectrophotometrically (Spektromon 204 spectrometer, Hungary) at 280 nm (the molar extinction coefficient of *E. coli* L-asparaginase is 9100). Cells with a thickness of 1 cm were used. Poliglyukin contents were determined by the Dubois method [12].

Disk electrophoresis in polyacrylamide gel was carried out by Davis's method [13]. Gel chromatography was performed in a column (17 × 240 mm, LKB, Sweden) containing Sephadex G-200 (Sweden) in 0.05 M K phosphate buffer, pH 8.0, and also in a column (12 × 300 mm, LKB) with the gel Toyopearl HW-55-Fine (Japan) in the same buffer.

To investigate the dependence of enzymatic activity on the pH of the medium we used 0.05 M universal buffer.

The spectrophotometric investigations were run on a Specord UV spectrophotometer (GDR). The depth of modification of the L-asparaginase by trinitrophenylation were determined as described in [14].

The antigenic affinities of the native and modified L-asparaginases were evaluated in the complement-binding reaction, which was performed as in [15] with the recording of the optical density of the liberated hemoglobin on a ABA-100 dichromate analyzer (USA) using a 450/415 spectral filter and a calibration factor of 1.0. Rabbit blood immune serum to native L-asparaginase was used in a dilution of 1/50.

The Binding of L-asparaginase with Poliglyukin. A solution of oxidized poliglyukin was added to a solution of 100 mg of L-asparaginase in 0.5 M K phosphate buffer, pH 8.5, at room temperature in an amount corresponding to the desired weight ratio of enzyme and polymer (see Table 1). The final volume of the reaction mixture was 30 ml. The binding reaction was carried out with continuous stirring and the maintenance of the pH of the medium in the interval of 8.2-8.5 for 3 h. Then the solution of immobilized L-asparaginase was cooled to 4 ± 2° and dry sodium tetrahydroborate (weight ratio of poliglyukin and sodium tetrahydroborate 3:1) was added in portions with continuous stirring until it had dissolved completely, and the mixture was then stirred for another 30 min. The subsequent ultrafiltration was carried out in FMOI-200 cells on "Ripor-4" membranes (Olaine, Latvian SSR) at a pressure of 2 atm. The volume after concentration was 30 ml. The final solution was lyophilized.

SUMMARY

The synthesis of *E. coli* L-asparaginase immobilized on a medical dextran — poliglyukin — has been effected.

The possibility and desirability of obtaining polymer conjugates of the enzyme with different amounts of poliglyukin have been shown.

The influence of the bound polymer on some physicochemical properties of the final products have been studied. An increased thermal stability and stability on storage of the immobilized form of L-asparaginase as compared with the native enzyme have been shown.

It has been found that the polymer modification of L-asparaginase leads to a decrease in the antigenic affinity of the immobilized enzymes as compared with the native enzyme.

LITERATURE CITED

1. A. D. Virnik, K. P. Khomyakov, and I. F. Skokova, Usp. Khim., 44, No. 4, 1280 (1975).
2. A. S. Karsakevich, S. N. Shulichenko, Yu. I. Shilov, and R. A. Zhagat, Khim. Prir. Soedin., 224 (1981).
3. French Patent Application No. 2059566/70, 2751, C/A61k 19/00/ (1970).
4. F. X. Hasselberger, H. D. Brown, S. K. Chattopadyay, A. N. Mather, R. O. Stasiw, A. B. Patel, and S. N. Pennington, Cancer Res., 30, 2736 (1970).
5. E. J. Benbough, C. N. Wiblin, and N. A. Raffer, Biochem. Pharmacol., 28, 833 (1979).
6. R. L. Foster and T. Wileman, J. Pharm. Pharmacol., 31, suppl., 37P (1979).
7. T. E. Wileman and P. N. C. Elliot, J. Pharm. Pharmacol., 33, suppl., 85 P (1981).
8. P. N. C. Elliot, R. L. Foster, and T. E. Wileman, Br. J. Pharmacol., 73, 303 P (1981).
9. P. N. C. Elliot, R. L. Foster, and T. E. Wileman, Br. J. Pharmacol., 74, 966 P (1981).
10. I. K. Shprunka, L. Ya. Binduka, T. V. Shidlovskaya, and R. A. Zhagat, Khim. Prir. Soedin., 231 (1980).
11. R. A. Zhagat, I. K. Shprunka, I. A. Éiduse, I. A. Vina, D. Ya. Daiya, Izv. Akad. Nauk Latv. SSR, Ser. Khim., No. 1, 73 (1972).
12. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, Anal. Chem., 28, 350 (1956).
13. B. J. Davis, Ann. N.Y. Acad. Sci., 121, 404 (1964).

14. L. Ya. Binduka, I. K. Shprunka, Yu. A. Benders, and R. A. Zhagat, Bioorg. Khim., 6, 1549 (1980).
15. O. A. Gorokhina, G. F. Leont'eva, and A. P. Kashkin, INÉI, No. 5, 111 (1979).