3. I. Cullen, in: Flora of Turkey and Eastern Aegean Islands, Vol. 1, P. H. Davis (ed.), Oliver and Boyd, Edinburgh (1965), p. 219.
4. I. Cullen, Flora Iranica, Graz, Austria (1966), p. 13.
5. S. Pfeifer and L. Kühn, Pharmazie, 23, No. 5, 267 (1968).
6. J. D. Phillipson, O. O. Thomas, A. I. Gray, and G. Sariyar, Planta Med., 42, No. 2, 105 (1981).
7. G. Sariyar and M. Shamma, Phytochemistry, 25, No. 10, 2403 (1986).
8. M. A. Manushakyan and V. A. Mnatsakanyan, Khim. Prir. Soedin., 713 (1977).
9. I. A. Israilov, S. U. Karimova, M. S. Yunusov, and S. Yu. Yunusov, Khim. Prir. Soedin., 279 (1980).
10. R. Ziyaev, A. Abdusamatov, and S. Yu. Yunusov, Khim. Prir. Soedin., 685 (1974).
11. J. Slavik, L. Slavikova, and L. Dolejs, Coll. Czech. Chem. Commun., 33, 4066 (1968).

IMMOBILIZATION OF THE ENZYME E. coli L-ASPARAGINASE ON A WATER-SOLUBLE COPOLYMER OF VINYLPYRROLIDONE AND ACROLEIN

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A method for immobilizing E. coli L-asparaginase on a copolymer of vinylpyrrolidone and acrolein has been developed and optimized. The influence on the nature of the modification of the number of acrolein residues in the copolymer has been established. The enzymatic and some physicochemical properties of the immobilized forms of the enzyme obtained have been studied.

We have previously reported the immobilization of the enzyme E. coli L-asparaginase, which is used in the treatment of a number of malignant systemic diseases of the blood, on various water-soluble polysaccharide carriers [1-3]. We now give the results of the immobilization of L-asparaginase on a synthetic water-soluble copolymer of vinylpyrrolidone and acrolein (CVA).

The interaction of L-asparaginase with the copolymer was based on the formation of an azomethine bond between the carbonyl groups of the CVA and the amino groups of the enzyme, as has been shown for the case of the modification of Terrilitin by this polymer [4]. To achieve stable links between the enzyme and the CVA, the reaction products were reduced with sodium tetrahydroborate, the free aldehyde groups of the polymer also being reduced by this reagent, as has been shown [5], and this, in the final account, imparted a neutral character to the carrier. In order to vary the electrochemical nature of the latter and to study its influence on the enzymatic activity, in individual cases reduction was also carried out with sodium bisulfite. This permitted the introduction of an additional negative charge into the polymer—protein conjugate [5].

The search for optimum conditions of synthesis ensuring the complete binding of the L-asparaginase to the CVA with the retention of a sufficiently high enzymatic activity revealed the importance of the pH of the medium and the length of the process of immobilizing the enzyme on the copolymer. Thus, the best results were obtained at pH $\geq$ 8 and a time of interaction of 3 h, i.e., under the conditions when the maximum amount of nonprotonated ε-amino groups of the lysine residues taking part in the reaction is probable.

The amount of CVA in the reaction mixture, i.e., the initial ratio of enzyme and polymer, also had a definite influence. The latter factor, in its turn, depended on the amount of acrolein residues in the samples of CVA.

The binding of L-asparaginase to the CVA led to an increase in the molecular weight of the enzyme. This was expressed in the chromatographic elution on Sephadex G-200 of the im-

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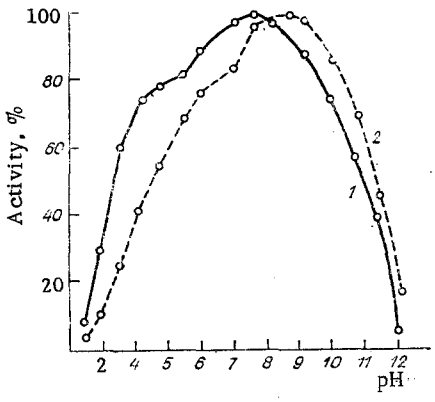

Fig. 1

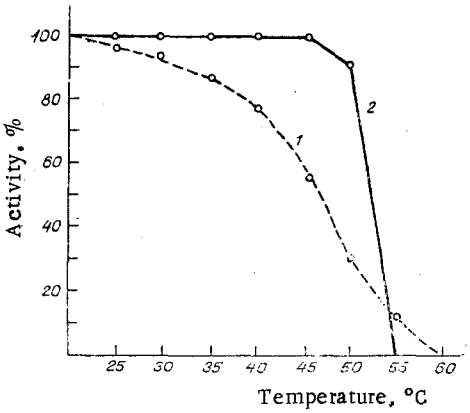

Fig. 2

Fig. 1. Dependence of the enzymatic activity on the pH of the medium: 1) native L-asparaginase; 2) 1:6 sample of L-asparaginase bound to the tetrahydroborate form of CVA (18 mole % of acrolein residues).

Fig. 2. Thermal stability of the native (1) and the immobilized (2) L-asparaginase.

TABLE 1. Degree of Modification and Enzymatic Activity of Immobilized L-Asparaginase

Index	Initial weight ratio of enzyme to CVA						
	1:3	1:5	1:6	1:7	1:8	1:9	1:10
Number of amino groups of enzyme modified by CVA containing the following amounts of acrolein residues:							
10 mole %	0	0	5,2	4,8	6.2	11,0	10.6
18 mole %	6,8	14,0	17,6	17,2	16,0	—	—
Percentage retention of the initial activity at the following contents of acrolein residues in the CVA							
10 mole %	—	—	57,1	69,9	70,4	21,0	28 5 30,2*
18 mole %	92,5	89,2	59,6 58,8*	62,6 63,4*	58.5	—	—

*In experiments with the bisulfite form of immobilized L-asparaginase.

mobilized samples with smaller retention volumes (K_{av} from 0.25 to 0.13) in comparison with those for the native enzyme (K_{av} 0.34). In agreement with this, on gel chromatography a decrease in the value of the distribution coefficients K_{av} of the polymer conjugates was observed. The presence of unbound L-asparaginase was expressed on the chromatogram by an additional protein peak with the mobility of the fraction characteristic for the native enzyme. When the L-asparaginase was modified with a copolymer containing 18 mole % of acrolein residues, beginning with a weight ratio of enzyme to copolymer of 1:6 the complete binding of the L-asparaginase was achieved. A further increase in the amount of CVA introduced into the reaction did not change the molecular weight of the final binding product or, consequently, the value of K_{av}.

Similar results were obtained in experiments with CVA containing 10 mole % of acrolein units. However, in this case complete binding of the components was observed only from initial weight ratio of enzyme to copolymer of 1:9 and above. Then, as shown in Table 1, an increase in the percentage amount of acrolein residues in the copolymer at any initial ratios of the components of the reaction mixture led to an increase in the number of points of binding between them. It is important to note that on the complete binding of L-asparaginase to CVA containing 18 mole % of acrolein residues (ratio of 1:6), in spite of the considerably larger number of modified amino groups of the enzyme, a larger percentage retention of enzy-

matic activity was observed than when the copolymer was modified with a smaller number of acrolein residues.

The results of the electrophoresis of immobilized samples of L-asparaginase likewise indicated the formation of high-molecular-weight interaction products, this being shown by the protein zones migrating with difficulty in the gel and their diffuseness, which is a characteristic indication of the electrophoretic behavior of polymeric derivatives of enzymes. Here, the electrophoretograms, in parallel with the results of gel chromatography with respect to the absence or presence of a zone of the native enzyme, permitted us to judge the completeness of the binding of the L-asparaginase to the CVA. Electrophoresis also permitted the revelation of the increase in the native charge of the polymeric conjugates of L-asparaginase that was assumed when sodium tetrahydroborate was replaced as reducing agent by sodium bisulfite. This was expressed electrophoretically in an increase in the mobility of the protein zone in the direction of the anode. At the same time, the changeover did not affect the enzymatic activity of the final product (Table 1), and it was necessary to elucidate this.

The study of the pH optimum of the catalytic action of the polymeric L-asparagine derivatives showed that modification caused a slight shift of the peak of the activity curve in the direction of alkaline values with only a slight change in the pH optimum of the enzyme (Fig. 1).

The immobilization of the L-asparaginase led to an increase in the thermal stability of the enzyme at temperatures up to 50°C. However, the stabilizing influence of immobilization disappeared sharply at temperatures above 55°C, and the thermal stability of the modified L-asparaginase under these conditions was even lower than that of the native enzyme (Fig. 2). Since the immobilized L-asparaginase is an enzyme for medical purposes, this should not cause misgivings, since at temperatures close to the physiological temperature it is more stable than the native L-asparaginase. In this case, the "low-temperature stabilization" of the enzyme characterized thermodynamically in [6] probably takes place.

A subsequent study of the biological properties of various forms of immobilized L-asparaginase will enable the most effective form of the enzyme to be selected.

EXPERIMENTAL

Highly purified *E. coli* L-asparaginase with a specific activity of 130 IU/mg of protein (Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR) was used.

The experimental samples of CVA (mean molecular weight 30,000 daltons) were obtained in the All-Union Scientific-Research Institute of Antibiotics and Enzymes for Medical Purposes (Leningrad), and the production of batches in the Biolar Scientific Production Combine (Olaine).

For gel chromatography we used a column of Sephadex G-200 (1.2 × 60 cm). Elution was performed with 0.05 M K-phosphate buffer, pH 8.0, at the rate of 0.2 ml/min. The distribution coefficients, K_{av}, were calculated in accordance with published methodological instructions [7]. Here the free volume was determined with the aid of dextran blue (Reanal).

The thermal stability of the samples was studied by incubating buffer solutions (10 IU/ml) at the appropriate temperatures for 1 h followed by the determination of the residual enzymatic activity. The activities of the native and immobilized L-asparaginases were determined by the method of direct Nesslerization [8] and the protein content by Lowry's method [9].

Disk electrophoresis in polyacrylamide gel was conducted by Davis's method [10]. The number of free primary amino groups in the immobilized L-asparaginase was determined by the trinitrophenylation method [11].

The Binding of L-Asparaginase to CVA. The copolymer (50 mg/ml) was dissolved in 0.01 N HCl and the solution was heated in the water bath for 1 h. Then the pH was brought to 8.0 with the aid of 0.01 N NaOH. The aqueous solution so obtained was treated with an equal volume of a solution of L-asparaginase in 0.01 M K phosphate buffer in such an amount as to establish the necessary weight ratio of enzyme to CVA in the reaction mixture. Binding was carried out at room temperature with moderate stirring for 3 h. The mixture was cooled to 4-5°C and the appropriate reducing agent – sodium tetrahydroborate or sodium bisulfite – was added in threefold molar excess. Reduction was performed at 4 ± 2°C with careful stirring for 1 h. Then the solution was subjected to ultrafiltration in FM01 cells of domestic production through XM-100 membranes (Amicon) at a pressure of 2 atm and was lyophilized.

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CONCLUSIONS

A method of immobilizing E. coli L-asparaginase on a copolymer of vinylpyrrolidone and acrolein has been developed and optimized. It has been found that the degree and nature of the binding of the enzyme to the polymer depend on the number of acrolein residues in the latter.

The absence of differences in the magnitude of the enzymatic activity of forms of immobilized L-asparaginase reduced with tetrahydroborate and by bisulfite has been shown. The influence of the bound copolymer on some physicochemical properties of the enzyme has been established. The immobilization of L-asparaginase leads to an increase in the stability of the enzymatic temperatures close to physiological.

LITERATURE CITED

1. O. B. Kinstler, A. S. Karsakevich, S. N. Shulichenko, and R. A. Zhagat, Khim. Prir. Soedin., 383 (1979).
2. A. S. Karsakevich, S. N. Shulichenko, Yu. I. Shilov, and R. A. Zhagat, Khim. Prir. Soedin., 224 (1981).
3. A. S. Karsakevich, S. N. Shulichenko, M. R. Buka, I. A. Vina, and A. P. Kashkin, Khim. Prir. Soedin., 766 (1986).
4. T. B. Tennikova, E. F. Panarin, O. A. Mirgorodskaya, G. V. Samsonov, and V. B. Moskvichev, Khim.-farm. Zh., No. 5, 86 (1977).
5. B. V. Moskvichev, Immobilized Enzymes in Medicine and the Medical Industry [in Russian], Leningrad (1982), p. 51.
6. G. D. Kutuzova, N. N. Ugarova, and I. V. Berezin, Usp. Khim., 53, No. 11, 1852 (1984).
7. Company Brochure: Sephadex. Gel Filtration in Theory and Practice, Pharmacia, Uppsala, Sweden (1970).
8. R. A. Zhagat, I. K. Shprunka, I. A. Éiduse, I. A. Vina, and D. Ya. Daiya, Izv. Akad. Nauk LatvSSR, Ser. Khim., 73 (1972).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., 193, 265 (1951).
10. B. J. Davies, Ann. N.Y. Acad. Sci., 121, 404 (1964).
11. L. Ya. Binduka, I. K. Shprunka, Yu. A. Benders, and R. A. Zhagat, Bioorg. Khim., 6, 1549 (1980).