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

I. COVALENT BINDING WITH INSOLUBLE SEPHAROSES AND CM-CELLULOSE

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UDC 577.155.3:547.458

Recently, special attention has been devoted to the questions of developing methods for obtaining and using immobilized enzymes in various branches of industry and medicine. Great interest in this connection is possessed by the enzyme L-asparaginase, which possesses antileukemic and immunodepressant properties [1].

Various polysaccharides and, in particular, cellulose and Sepharose are used very frequently for immobilization. The possibility of using insoluble cellulose derivatives for the immobilization of L-asparaginase has already been shown in a number of investigations [2-4].

The present paper gives the results of the chemical binding by various methods of the enzyme L-asparaginase to Sepharose 4B and its derivatives AH-Sepharose 4B and CH-Sepharose 4B and also to CM-cellulose and reports some properties of the preparations obtained.

The results of the chemical binding of L-asparaginase to the polysaccharides mentioned are given in Table 1.

The binding of L-asparaginase to BrCN-activated Sepharose 4B was practically complete in 2 h with the addition of the maximum possible amount of enzyme to the support (Fig. 1). At the same time, as can be seen from the figures given below, the highest amount of added enzyme did not always give the highest yield of activity in the immobilized product, which can be explained to some extent by the sterically hindered approach of the substrate to the active center of the added enzyme at high concentrations of the latter [5]:

Amount of Added L-Asparaginase		Activity of the Immobilized L-Asparaginase	
IU/g of gel	mg/g of gel	IU/g of gel	%
2000	11.11	800	40
3000	16.67	936	31.2

Of the other supports, the best results were obtained by binding L-asparaginase by the carbodiimide method with CH-Sepharose 4B and by the glutaraldehyde method with AH-Sepharose 4B. At the same time, the carbodiimide method of coupling the carboxy groups of the enzyme with the latter polysaccharide gave a very low result in respect of activity. In this case, the polycondensation of the molecule of the L-asparaginase itself and the modification of some of its functional groups probably take place. The glutaraldehyde and cyanogen bromide methods give good results both in relation to the amount of enzyme added and in relation to the degree of activity thanks to the presence of a considerable number of lysine ε-amino groups in the L-asparaginase molecule [6], which participate in coupling with the support.

A characteristic of the immobilized L-asparaginase preparations is their high stability on storage and their heat resistance in comparison with the free enzyme. Figure 2 shows, for a number of preparations as examples, the stability of the immobilized L-asparaginase that is characteristic for all modifications on polysaccharide supports.

Native L-asparaginase retains its activity over a wide pH range with the optimum pH at 7-8 [7]. The curve of the activity of L-asparaginase bound to polysaccharide supports contracts somewhat. There is usually

Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 639-643, September-October, 1976. Original article submitted May 12, 1976.

TABLE 1. Characteristics of the Insoluble Preparations Obtained by Chemical Methods of Coupling L-Asparaginase with Some Polysaccharides

Support	Amount of added L-asparaginase, IU/g of preparation				L-asparaginase activity, IU/g of preparation				Activity yield, %			
	G	C	S	CB	G	C	S	CB	G	C	S	CB
Sepharose 4B	3780	700	400	2000	872	3,8	67	800	23,3	0,54	17	40
AH-Sepharose 4B		1000				254,0				25,40		
CH-Sepharose 4B		2290				138,4				9,30		
CM-Cellulose												

Note. G) glutaraldehyde method; C) carbodiimide method; S) succinimide method; CB) cyanogen bromide method.

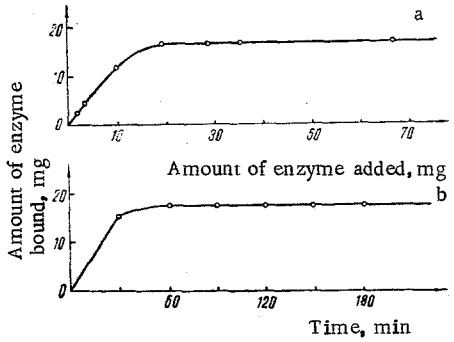

Fig. 1. Dependence of the amount of L-asparaginase bound (a) on the amount of protein added and (b) on the time of the reaction with the optimum amount of added enzyme.

no displacement of the pH optimum or it is insignificant although at the same time a more pronounced narrow activity peak is observed (Fig. 3).

One of the strongest competing inhibitors of native L-asparaginase, N-benzyloxycarbonyl-S-benzyl-L-cysteine, in a concentration of $3 \cdot 10^{-3}$ M inhibits the activity of the native enzyme by 95%, and that of the matrix-attached enzyme to a considerably smaller degree (see below).

A statistical analysis of the results of the dependence of the activity of immobilized preparations of L-asparaginases showed their agreement with the Michaelis–Menten equation, although the values of the apparent Michaelis constant (K_{Mapp}) are somewhat greater. This can be explained to some extent by the diffusion of the substrate, limiting the rate of the whole process, and by possible conformational changes of the support-bound enzyme. The values of the kinetic constant K_{Mapp} of some bound L-asparaginases obtained by linear regression after the transformation of the results by the Lineweaver–Burk method are as follows:

L-Asparaginase	K_{Mapp}, M	Inhibition by $3 \cdot 10^{-3}$ M N-Benzyloxycarbonyl-S-benzyl-L-cysteine, %
Bound to Sepharose 4B	$5.2 \cdot 10^{-5}$	15
Bound to AH-Sepharose 4B (glutaraldehyde method)	$8.9 \cdot 10^{-5}$	1
Native	$1.16 \cdot 10^{-5}$	95

EXPERIMENTAL

A purified preparation of the enzyme – L-asparaginase from E. coli (material from the Riga medical preparations factory) – with a specific activity of 180 IU/mg was used. The supports were Sepharoses from the Swedish firm Pharmacia: Sepharose 4B activated with BrCN, CH-Sepharose 4B activated with N-hydroxysuccinimide, CH-Sepharose 4B, AH-Sepharose 4B, and Whatman CM-cellulose.

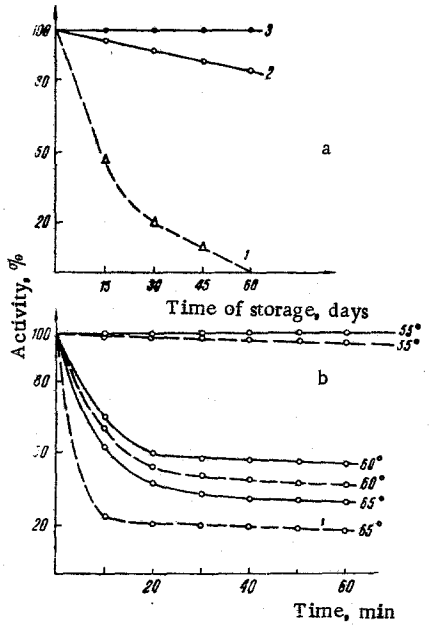

Fig. 2

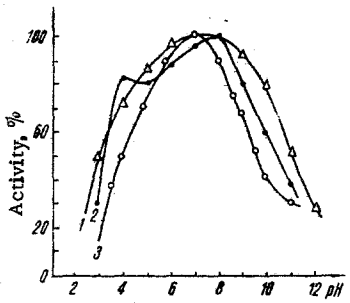

Fig. 3

Fig. 2. Stability of immobilized L-asparaginase: a) stability on storage of the native enzyme (1) and of the enzyme immobilized on CH-Sepharose 4B (2) and on Sepharose 4B (3): b) heat stability of the native enzyme (broken line), and of the enzyme immobilized on AH-Sepharose 4B by the glutaraldehyde method of coupling (full line).

Fig. 3. Dependence of the activity on the pH of the medium for native L-asparaginase (I) and for L-asparaginase immobilized on CH-Sepharose 4B (2) and on Sepharose 4B (3).

All the immobilized preparations of L-asparaginase were stored in the form of aqueous suspensions at 5°C.

The activities of the soluble and insoluble L-asparaginases were determined as described previously [8, 9]. To determine the dependence of the activity of the insoluble L-asparaginase on the pH of the medium we used 0.05 M universal buffer (pH 2-12) [10].

Binding of the L-Asparaginase to Sepharose 4B. The enzyme was bound to BrCN-activated Sepharose 4B (amounts given above) by Porath's method [11]. The final product was washed on the filter with water, 0.5 M NaCl, and water again.

Glutaraldehyde Method of Coupling L-Asparaginase with AH-Sepharose 4B. A suspension of 0.5 g of AH Sepharose 4B in 5 ml of water was treated with 3 ml of a 25% aqueous solution of glutaraldehyde and the mixture was stirred for 1 h. After filtration, the support was carefully washed with water (~ 10 times).

The activated support was suspended in 5 ml of water containing 10 mg of L-asparaginase, the mixture was stirred for 2 h, and the product was filtered off. Then it was washed with water, 1 M NaCl solution, and water again.

The Carbodiimide Method of Coupling L-Asparaginase with AN Sepharose 4B. To 4 ml of a solution of the enzyme (5 mg) was added 200 mg of 1-cyclohexyl-3-[2-(4-methylmorpholinio)ethyl]-carbodiimide p-toluenesulfonate (CMC). Then it was mixed with 0.5 g of AH-Sepharose 4B suspended in water. The reaction was performed at room temperature, pH 5.0-5.7, for 10 h, the pH being adjusted where necessary with dilute solutions of HCl and NaOH. The final product was carefully washed with water, 1 M NaCl, and water again.

Carbodiimide Method of Coupling L-Asparaginase with CH-Sepharose 4B. A suspension of 0.5 g of the support in 5 ml of water was treated with 100 mg of CMC, and the mixture was stirred at pH 4.7-5.0 for 15 min. Then 5 mg of L-asparaginase was added and mixing was continued at room temperature for 6 h in the above-given pH range. After filtration, the product was carefully washed with water, 1 M NaCl, and water again.

The Succinimide Method of Coupling L-Asparaginase with CH-Sepharose 4B. To a suspension of 1 g of activated succinimide ether of CH-Sepharose 4B in 10 ml of K phosphate buffer with pH 8.0 was added 10 mg of L-asparaginase, and the mixture was stirred at room temperature for 1.5 h. Then the insoluble product were filtered off and was washed with K phosphate buffer, 1 M NaCl, and water.

Carbodiimide Method of Coupling L-Asparaginase with CM-Cellulose. By the method of Nikolaev and Mardashev [2], 500 mg of CMC was added to a suspension of 2 g of CM-cellulose in 30 ml of 0.1 M HCl, and the mixture was stirred at room temperature for 3 h. Then the product was filtered off and washed with water.

The activated CM-cellulose was suspended in 10 ml of water, 25 mg of L-asparaginase was added, and the mixture was stirred at 4°C for 16 h. The insoluble enzyme was washed on the filter with water, 1 M NaCl, and water again.

SUMMARY

Immobilized L-asparaginase has been synthesized by various methods of covalent binding to insoluble Sepharoses and CM-cellulose.

Some kinetic properties of the preparations obtained, the dependence of the activity on the pH of the medium, and their stability have been investigated.

It has been established that the immobilized L-asparaginase possesses increased heat resistance, stability on storage, and stability to competing inhibitors.

LITERATURE CITED

1. R. L. Capizzi and R. E. Handschumacher, "Asparaginase," in: Cancer Medicine, Philadelphia (1973), p. 850.
2. A. Ya. Nikolaev and S. R. Mardashev, Biokhimiya, 26, 641 (1961).
3. F. X. Hasselberger, H. D. Brown, S. K. Chattopadhyay, A. N. Mather, R. O. Stasiw, A. B. Patel, and S. N. Pennington, Cancer Research, 30, 2736 (1970).
4. A. Ya. Nikolaev, Biokhimiya, 27, 843 (1962).
5. Pharmacia Brochure. CNBr-Activated Sepharose 4B for the Immobilization of Biopolymers, Uppsala (1974), p. 10.
6. J. Nishimura, H. Makino, and O. Takenaka, Biochem. Biophys. Acta, 227, 171 (1971).
7. P. P. K. Ho, E. B. Milskin, J. L. Bobbitt, E. L. Grinnau, P. Z. Buren, B. H. Frank, U. D. Borck, and R. W. Squire, J. Biol. Chem., 245, 3708 (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. A. S. Karsakevich, G. Ya. Migla, and R. A. Zhagat, Prikl. Biokhim. Mikrobiol., 11, 773 (1975).
10. Yu. Yu. Lur'e, Handbook on Analytical Chemistry [in Russian], Moscow (1962).
11. R. Axen, J. Porath, and S. Ernback, Nature, 214, 1302 (1967).

REDUCTIVE CLEAVAGE BY SODIUM IN LIQUID AMMONIA OF THE LIGNIN OF COTTON-PLANT STEMS

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UDC 547.458.84+549.927.2

Reductive cleavage by alkali metals in liquid ammonia is widely used to study the structural links in the lignin molecule and the bonds between them [1-8]. 1-(4-Hydroxy-3-methoxyphenyl)propane, 1-(4-hydroxy-3-methoxyphenyl)propan-1-ol, and 1-(4-hydroxy-3,5-dimethoxyphenyl)propane have been identified previously by paper chromatography in the products of the cleavage of cotton-plant stem lignin [9].

We have investigated the reductive cleavage by sodium in liquid ammonia of the lignin of the green stems of the cotton plant of the variety 108-F before their treatment with ultrasound and after it. In the two cases

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 643-646, September-October, 1976. Original article submitted May 20, 1976.