L-ASPARAGINASE ENTRAPPED IN LIPOSOMES: PREPARATION AND PROPERTIES

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1. Introduction

L-Asparaginase has been used with remarkable success in the treatment of certain forms of leukemia [1,2]. However, a major drawback in its continued clinical application has been the frequently observed occurrence of hypersensitive reactions to the enzyme as a foreign protein [3,4].

In principle it should be possible to prevent or at least minimize the immunogenic effect of repeated administration of the enzyme by entrapping it within non-antigenic particles. The packaging of L-asparaginase in microcapsules which are immunologically inert has been described before [5,6]. However, the objection has been raised that such microcapsules contain undegradable material which will accumulate in the body. The alternative approach of using liposomes [7] as inert vehicles [8] for L-asparaginase is outlined in this communication. We describe the preparation and some of the properties of asparaginase-carrying liposomes. Of particular interest is the observation that in such liposomes the antigenic determinants of L-asparaginase are completely masked. The evaluation of the asparagine-liposomes in vivo is now in progress.

2. Materials and methods

L-Asparaginase from *E. coli* (EC 3.5.1.1, L-asparagine amidohydrolase) was a gift from Merck, Sharp and Dohme. Egg phosphatidylcholine was donated by Dr S. Gatt of the Department of Biochemistry, Medical School, Jerusalem. It was prepared in his laboratory as by Pangborn [9]. Dicetyl phosphate was purchased from Sigma Chemical Company, cholesterol and L-asparagine from Nutritional Biochemical Corporation. Trypsin was obtained from Worthington Biochemical Corporation.

2.1. *Preparation of liposomes*

Liposomes were prepared as by Gregoriadis et al. [7] with slight modifications. Egg phosphatidylcholine (40 μmol), cholesterol (8 μmol) and dicetyl phosphate (4 μmol) were dissolved in 2 ml of chloroform in a 50 ml round-bottomed flask. The chloroform was evaporated by rotation under nitrogen. The thin film which formed on the walls of the flask was dispersed in 2 ml of 5 mM potassium phosphate buffer (pH 7.5) containing asparaginase (150 I.U.). Aspartic acid (20 μmol) and gelatin (2.5 mg) were added to stabilize the enzyme [10]. The liposomes formed by shaking for 2 h at room temperature were washed twice in saline at 100 000 *g* for 60 min and resuspended in saline. 'Empty' liposomes were prepared as above in the absence of asparaginase.

2.2. *Assay of asparaginase*

Enzyme assays were carried out at 37°C in 2 ml Tris–HCl buffer (0.05 M, pH 8.0) containing 2 μmol of L-asparagine as described elsewhere [10]. The liberated ammonia was determined by direct nesslerization and the optical density measured at 420 nm in a Klett-Summerson colorimeter. One unit of enzyme releases 1 μmol of ammonia in 1 min at 37°C and pH 8.0.

2.3. *Heat treatment and assay of residual asparaginase activity*

The treatment was carried out in test tubes immersed in a water bath with the temperature regulated by a Thermomix II immersion thermostat. Samples of the enzyme were incubated at the desired temperature in

0.5 ml of Tris–HCl buffer (0.04 M, pH 8), unless otherwise indicated. The treatment was terminated at various time intervals by immersing the test tubes in an ice bath for 1 min. The assay of residual activity was started by adding 1.5 ml of the substrate solution prewarmed to 37°C.

2.4. *Trypsin treatment*

Proteolysis was carried out with trypsin (20 μg/ml) at 37°C in 0.5 ml of Tris–HCl (0.04, pH 8.0). A freshly prepared solution of trypsin in 0.01 M $CaCl_2$ was used in each experiment. The treatment was terminated by the initiation of the assay as described above.

2.5. *Preparation of antisera*

Antisera were prepared in rabbits by intramuscular injection of 20 I.U. of L-asparaginase homogenized with complete Freund's adjuvant. Injections were repeated 4 times at 10-day intervals and the rabbits were bled from the ear 10 days after the last injection. The blood was allowed to clot overnight and the serum was separated and stored at −20°C. A month after the last bleeding, the rabbits were given a booster injection of 20 I.U. of the enzyme. The sera were collected 10 days later and stored as above. All sera were pooled before use.

2.6. *Neutralization and stabilization by antibody*

Samples of the enzyme were incubated for 5 min at 37°C with varying amounts of antiserum and 40 μmol Tris–HCl (pH 8.0) in a total vol of 0.5 ml made up with normal rabbit serum. To measure stabilization the sample was heat-treated before the residual activity was determined. The procedure of treatment and assay was as described above.

3. Results and discussion

The efficiency of entrapment of L-asparaginase in liposomes compares favourably with that reported for other enzymes [7]. Thus, under the conditions described in Materials and methods, over 12% of the original enzyme activity was retained in the washed liposome preparation. Several runs with altered proportions of the lipid components resulted in considerably lower yields. Intact, washed liposomes showed about 42% of the activity of the entrapped enzyme, and full activity was recovered after disruption of the liposomes by osmotic shock.

Entrapment of L-asparaginase in liposomes results in a marked increase in the thermostability of the enzyme. There is no increase in thermostability of the enzyme when added to a pre-formed enzyme-free liposome preparation. The thermostability is lost when the liposomes are suspended in distilled water and osmotically disrupted (table 1).

Enclosure of L-asparaginase in liposomes similarly protects the enzyme against proteolytic inactivation. As shown in fig.1, incubation of free L-asparaginase with trypsin (20 μg/ml) leads to rapid loss of activity

Table 1
Thermostability of entrapped L-asparaginase

	Temperature	Asparaginase activity remaining after heating[a]			
		1	2	3	4
		(min)			
Intact liposomes	68°C	81.7	88.7	80.3	74.6
	70°C	82.0	66.3	55.1	55.1
Lysed liposomes	68°C	7.3	6.3	7.3	5.2
	70°C	3.4	2.5	–	2.5
Free enzyme	68°C	4.2	1.6	1.6	2.1
	70°C	1.1	0	0	0

[a] Asparaginase activity (I.U./ml) remaining after exposure to 68°C or 70°C was determined as described in Materials and methods.

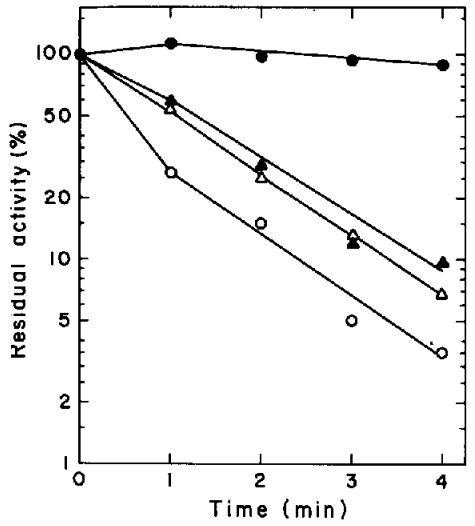

Fig.1. Resistance of entrapped L-asparaginase to proteolysis. The residual activity of enzyme preparations incubated at 37°C with trypsin (20 μg/ml) was assayed at the indicated time intervals. For other details see Methods. (●) Intact asparaginase-liposomes; (○) lysed asparaginase-liposomes; (△) asparaginase + empty liposomes; (▲) asparaginase alone.

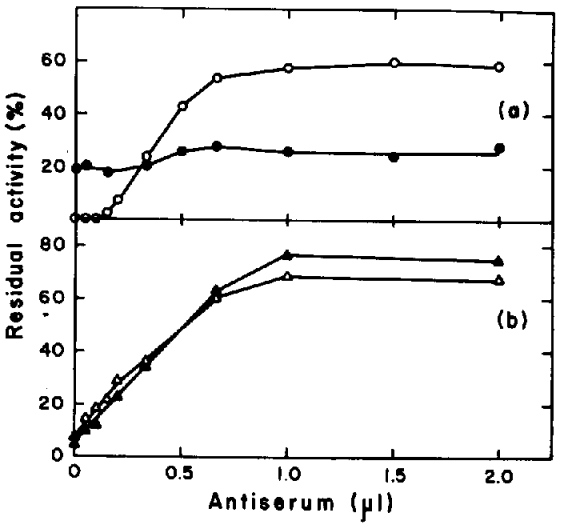

Fig.2. Entrapped L-asparaginase is not stabilized by antibodies to the free enzyme. The enzyme preparations were pre-incubated at 37°C for 5 min with the indicated amounts of antiserum (see Materials and methods) and subsequently exposed to (a) 70°C or (b) 62°C for 7 min. The residual activity was then determined as described in Materials and methods. For symbols see fig.1.

of 37°C, whereas the entrapped enzyme is completely resistant to proteolysis under the same conditions. The resistance to proteolysis is lost when the liposomes are disrupted by osmotic shock (fig.1).

These results indicated that the entrapped enzyme is effectively sealed within the liposomal membranes and thus inaccessible to trypsin. Consistent with this was the further observation that the liposome-bound asparaginase does not react with antibodies prepared against the free enzyme. It has been shown [11] that

Table 2
Effect of antiserum against asparaginase on the activity of free and entrapped enzyme

	Antiserum (μl)			
	0	0.66	1.0	2.0
	Residual activity of enzyme (I.U./ml) after incubation 10 min at 37°C with antiserum			
Intact asparaginase-liposomes	0.57	0.58(0)	0.59(0)	0.59(0)
Lysed asparaginase-liposomes	0.49	0.41(0.17)	–	0.36(0.27)
Empty liposomes + asparaginase	1.37	1.12(0.18)	1.09(0.21)	1.00(0.27)
Asparaginase alone	1.25	1.15(0.08)	1.02(0.18)	0.93(0.26)

Figures in brackets represent fraction of activity neutralized by antibodies.

the antibodies neutralize to some extent the activity of the free enzyme. There is, however, no neutralization of asparaginase entrapped in intact liposomes (table 2). A more sensitive indicator of the interaction of L-asparaginase with the homologous antibodies is provided by the observation [11] that the enzyme-antibody complex is stabilized against thermal inactivation. The effect of increasing amounts of antibodies on the thermostability of L-asparaginase is shown in fig.2. The stabilization obtained with the free enzyme and with disrupted liposomes or with a mixture of the enzyme and 'empty' liposomes is not observed when the enzyme is entrapped in liposomes. It appears therefore that the antigenic determinants of this enzyme are effectively masked by the entrapment. Consequently, intact liposomes can be considered as antigenically inert vehicles for this enzyme. The behaviour in vivo [12] and the therapeutic potential of asparaginase-liposomes is presently under evaluation.

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