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Complexing of Basic Pancreatic Proteinase Inhibitor with Soybean Phospholipid Multilamellar Vesicles

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Abstract—The formation of complexes of basic pancreatic proteinase inhibitor (BPTI) with multilamellar vesicles (MLV) from six preparations of soybean phospholipids of various composition was studied. When BPTI, a non-membrane protein, interacts with MLV, the vesicles aggregate, forming a precipitate of protein–lipid complexes. The BPTI content in the protein–lipid complexes increases with decreasing pH of the medium and on addition of negatively charged components into the lipid mixture. The protein-induced aggregation of the phospholipid vesicles is suggested to be mainly determined by electrostatic forces. The antiproteinase activity of BPTI in the complexes was rather low but increased up to 70% of the initial activity on addition of an ionic detergent (sodium deoxycholate).

Key words: protein–lipid interaction, multilamellar phospholipid vesicles, BPTI, inhibiting activity

The interaction of proteins with phospholipids and their functioning in the resulting complexes has been intensively studied for many years [1]. Studies in this field have been most successful in the case of membrane proteins [2] and also of water-soluble proteins that bind to membranes during their functioning or transfer to their site of localization [3]. But studies on soluble proteins not functionally connected with membranes is another case. However, it is obvious that these proteins function in intercellular space or in cytosol, i.e., in a non-homogenous environment that has a certain structure. Studies on microheterogenous systems such as reversed micelles with an organic solvent for the dispersion medium and water for the disperse phase have shown significant changes in the properties of these proteins compared to a homogenous aqueous phase [4]. The structure of a model membrane system consisting of an aqueous dispersion medium and phospholipid aggregations (liposomes, multilamellar vesicles) for the disperse phase is more like the structure of biomembranes. This system was used for studies on the ability of soluble non-membrane proteins (lysozyme, trypsin, dehydrogenase) to induce aggregation of phospholipids [5, 6]. However, the functional state of these proteins in the complexes was not studied.

In the present work the complexing of a water-soluble protein, basic trypsin inhibitor (BPTI), with various lipid preparations and also the inhibitor activity in the protein–lipid complexes were studied.

MATERIALS AND METHODS

The following reagents were used: Gordox preparation of BPTI (Gedeon Richter, Hungary) desalinated on Sephadex G-25 and freeze-dried, containing 54% of the active inhibitor; phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) of 98% purity (Lipoid, Germany); sodium deoxycholate (SDC) (Merck, Germany); solvents of chemical purity (Khimmed, Russia). Soybean lipid extract LP-1 was prepared from a half-defatted soybean meal (GOST 3898-56, Assoya, Krasnodar, Russia) as described in [7]. Preparation of lipid extracts LP-2 and LP-3 and also methods of determination of their composition were described earlier [8]. The individual phospholipids phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylglycerol (PG) were isolated from LP-3 by absorption chromatography on silica gel

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using a chloroform–methanol system with methanol concentration gradient from 10 to 30%. The phospholipids were used to prepare mixtures LP-4 and LP-5.

Preparation of BPTI–multilamellar phospholipid vesicle complexes. Multilamellar vesicles (MLV) of various lipid preparations were obtained as follows: the lipids dissolved in chloroform were evaporated to dryness under vacuum, the lipid films were dispersed in aqueous medium at two pH values: 3.0 (0.001 M HCl) and 8.0 (0.05 M Tris-HCl buffer). To prepare complexes, 1 ml of aqueous dispersions of phospholipids (3 mg/ml) was incubated for 10 min with 0.05 ml of BPTI solution at pH 3.0 or 8.0. The resulting precipitates were separated by centrifugation at 60*g* for 10 min and washed twice with 0.001 M HCl and 0.05 M Tris-HCl buffer, respectively. Then the precipitates were dispersed in 1 ml of 0.05 M Tris-HCl buffer (pH 8.0). The size of the MLV of every preparation and of the protein–lipid complexes was determined by turbidimetry and by quasi-elastic light scattering (N4 Coultronics, USA) [9, 10]. The mean hydrodynamical diameter of the vesicles was calculated using the Stokes–Einstein equation on the assumption that the MLV particles were independent and spherical.

Composition of protein–lipid complexes. To determine the composition of complexes prepared by precipitation and redispersion, phospholipid contents were determined as described in [11] and the protein content was determined by a modified Lowry method [12].

Determination of antitryptic activity of BPTI–lipid complexes. Trypsin solution (0.1 ml, 0.1 mg/ml) in 0.001 M hydrochloric acid was placed in a 1 ml spectrophotometer cuvette, then 0.015-0.030 ml of the BPTI–lipid complex solution (0.02-0.07 mg protein/ml) was added and the total volume of the mixture was adjusted to 0.8 ml with 0.05 M Tris buffer (pH 8.0). The mixture was incubated for 7 min at room temperature. Then the substrate solution of N-benzoyl-L-arginine ethyl ester (0.2 ml, 1.5 mM) in 0.05 M Tris-HCl buffer (pH 8.0) was added into the cuvette. Changes in absorption of the solution were recorded at 253 nm using a Shimadzu UV-265 FW spectrophotometer (Japan). The rate of the substrate hydrolysis by trypsin (0.1 mg/ml) was determined simultaneously in the presence and in the absence of individual phospholipids to ascertain the amount of trypsin bound to them under the experimental conditions.

Determination of antitryptic activity of BPTI–lipid complexes in the presence of 0.5% SDC. Aqueous dispersions of BPTI–lipid complexes (0.02 ml, 0.2-0.7 mg protein/ml) were incubated with 0.02 ml 5% SDC solution for 0.5 h, then the mixture was diluted fivefold and 0.03 ml was taken for determination of BPTI activity in the complex (as described earlier). The rate of the substrate hydrolysis by trypsin (0.1 mg/ml) was determined simultaneously in the presence and in the absence of individual lipids and SDC to ascertain the amount of trypsin bound to the phospholipids under the conditions and also the amount of trypsin that lost activity in the presence of SDC. The quantity of active BPTI in the complex in the presence of SDC was calculated taking into account the effect of the detergent on the native protein.

RESULTS AND DISCUSSION

Choice of the model system. BPTI is a basic protein (p*I* = 10.5) [13]. Therefore, to obtain complexes with BPTI a soybean lipid preparation (LP-1) was chosen that contained phospholipids of various classes: zwitterions (PC and PE), negatively-charged phospholipids (PI and PG), and lysocomponents (LPC) (Table 1). The phospholipid composition of LP-1 determined earlier [8] is characteristic of membranes of eucaryotes. In addition to phospholipids, LP-1 also contained fatty acids, saponins, and minor quantities of glycolipids, sterines, and proteins. Because of the complicated composition of the initial preparation, a number of lipid preparations of simpler composition (from LP-2 to LP-6) was obtained from it by successive purification to be used as controls. Note that the ratios of individual phospholipids in the multicomponent mixtures were rather close to the ratios in the initial preparation LP-1 (Table 1).

Table 1. Composition of lipid preparations

Preparation	Phospholipid content, % by weight	Other components, % by weight			Individual phospholipids, % of total phospholipids				
		fatty acids	saponins	proteins	PC	PE	PI	PG	LPC
LP-1	59 ± 3	8 ± 1	30 ± 2	4.5 ± 0.5	42 ± 2	20 ± 2	18 ± 2	13 ± 1	7 ± 1
LP-2	87 ± 3	11 ± 1	—	3.5 ± 0.3	42 ± 2	20 ± 2	18 ± 2	13 ± 1	7 ± 1
LP-3	98 ± 2	—	—	—	56 ± 2	20 ± 2	8 ± 1	8 ± 1	7 ± 1
LP-4	98 ± 2	—	—	—	44 ± 2	22 ± 2	34 ± 2	—	—
LP-5	98 ± 2	—	—	—	67 ± 3	33 ± 2	—	—	—
LP-6	98 ± 2	—	—	—	98 ± 2	—	—	—	—

First, the LP-1 preparation was treated with 0.1 M NaCl to remove water-soluble admixtures (saponins); thus, the LP-2 preparation contained fatty acids and minor components in addition to phospholipids. They were removed by adsorption chromatography to produce the LP-3 preparationsa multicomponent phospholipid mixture containing PC, PE, PI, PG, and LPC. Then from the individual phospholipids obtained from LP-3 by adsorption chromatography a two-component LP-5 (PC : PE) mixture and a three-component LP-4 (PC : PE : PI) mixture were prepared. The simplest system was an aqueous dispersion of PC (LP-6) that was used as a control.

Table 1 shows that the studied lipid preparations isolated from soybean lipids or prepared from individual phospholipids were different by one component, and this allowed us to determine the effect of each of the components.

Preparation of complexes and analysis of their composition. Due to protein-induced aggregation of MLV, the protein–lipid complexes were separated from the free lipids and protein. To choose optimal conditions for the precipitation, the dependence of the phospholipid and protein contents in the precipitate on the initial phospholipid concentration was determined for the case of LP-1 (Fig. 1). The figure shows that the precipitation was maximal at initial phospholipid concentration 3 mg/ml at both pH values (3.0 and 8.0). Note that the phospholipid concentration means not the concentration of lipid preparations but just the concentrations of phospholipids in these preparations. Precipitates obtained at different pH values were redispersed for the further studies only at pH 8.0 because we failed to fully redisperse them at pH 3.0. Polyacrylamide gel electrophoresis [14] of the complexes prepared at the two pH values and with various lipid preparations showed that the complexes were not destroyed during redispersion.

Data on the composition of the complexes are presented in Table 2. For all preparations except LP-6 (PC) and at both pH values the phospholipids were virtually completely included in the complexes, whereas PC slightly complexed with BPTI only at pH 3.0. Unlike the phospholipid contents, the protein content in the complexes depended on both the pH of the medium and the phospholipid preparation used. Some conclusions can be drawn from the data presented in Table 2. First, the amount of protein that complexed with the phospholipids was in all cases higher at pH 3.0 than at pH 8.0, this probably being due to the higher net positive charge on the protein molecule (pI = 10.5) in the acidic medium. This is associated with a decrease in the total negative charge of acidic phospholipids. Because the molar quantities of acidic phospholipids in the studied protein–lipid complexes were much higher than that of the protein (Table 2), the decrease in the negative charge on the surface of the phospholipid aggregates on increasing the pH from 3.0 to pH 8.0 could be neglected. Based on these findings, the binding forces are suggested to be electrostatic. Such interaction could occur between positively charged amino acid residues of protein molecules (e.g., lysine and arginine) and negatively charged phospholipid heads.

Second, as mentioned above, PC interacted only weakly with BPTI at pH 3.0 and did not produce a precipitable complex at pH 8.0. The presence of acidic phospholipids in the mixture increased the protein and phospholipid contents in the precipitate at both pH values. Thus, addition of PI into the PC : PE : PI mixture (LP-4) resulted in a twofold increase in the BPTI content in the complex compared to the PC : PE mixture (LP-5). The protein content was the highest (70%) in the complex with the LP-1 preparation, which differed from the other lipid preparations by the presence of saponins and fatty acids. The literature data suggest that the glycoside part of soybean saponins contains glucuronic acid residues [15].

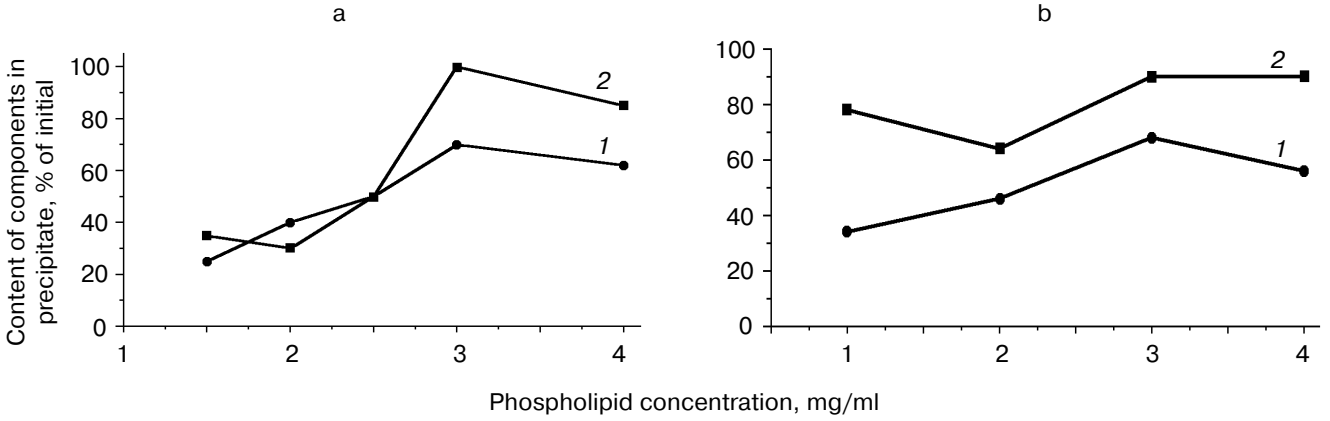

Fig. 1. Contents of BPTI (*1*) and phospholipids (*2*) in the precipitated complex depending on the initial concentration of phospholipids in the LP-1 preparation at pH 3.0 (a) and 8.0 (b). Protein concentration, 1 mg/ml.

Table 2. Composition of precipitated BPTI–lipid complexes ([LP] = 3 mg/ml, [BPTI] = 1 mg/ml)

Preparation	Precipitated as complex, wt. % of initial				Phospholipid/BPTI in complex, mole/mole	
	phospholipids		BPTI			
	pH 3.0	pH 8.0	pH 3.0	pH 8.0	pH 3.0	pH 8.0
LP-1	96 ± 2	96 ± 3	70 ± 3	62 ± 5	30 : 1	38 : 1
LP-2	96 ± 3	96 ± 1	60 ± 4	52 ± 2	40 : 1	50 : 1
LP-3	98 ± 2	94 ± 3	50 ± 2	26 ± 3	50 : 1	80 : 1
LP-4	97 ± 3	96 ± 2	65 ± 5	37 ± 2	30 : 1	60 : 1
LP-5	95 ± 4	93 ± 2	30 ± 2	10 ± 3	80 : 1	240 : 1
LP-6	68 ± 3	n.c.	10 ± 3	n.c.	120 : 1	n.c.

Note: n.c., no complex produced.

Removal of saponins from the lipid extract was accompanied by a decrease in the BPTI content in its complex with the LP-2 preparation. The LP-2 preparation, in turn, was different from the multicomponent phospholipid mixture LP-3 by the presence of fatty acids and was characterized by increased binding of the protein (60 and 50%, respectively). Thus, with an increase in the relative content of negatively charged components in the preparations, the content of BPTI in the complexes increased, and this supports the above-mentioned hypothesis about the electrostatic interaction between the protein and phospholipid soybean preparations.

Third, the addition of PE to PC resulted in a nearly threefold increased amount of BPTI precipitated in the complex. Moreover, if the content of PE in the lipid mixture was lower than 20% (data not presented), no precipitable complex with the protein was produced. This finding was somewhat unexpected because PE is a zwitterion similar to PC. Its addition into the phospholipid mixture did not change its charge. Nevertheless, the protein was significantly bound to this lipid preparation, indicating that the complexing depended on various factors and not only on electrostatic interactions. The effect of PE suggested a significant role of hydrate envelopes of the charged groups in phospholipid molecules during complexing: the lower hydration of PE compared to PC [16] promoted the complexing.

Activity of the inhibitor in complexes. Based on data on the composition of the precipitated complex, the specific activity of the inhibitor was calculated in the complexes prepared under different conditions. BPTI was the least active in the complexes 1 h after their preparation (Fig. 2). On addition of the ionic detergent SDC (0.5%) to BPTI–lipid complexes, the inhibitor activity significantly increased in all samples. And the inhibitor activity tended to be higher in the protein–lipid complexes prepared at pH 8.0 (Fig. 2). Electrophoresis of complexes incubated in the presence of SDC showed no release of free inhibitor in dispersions of the protein–lipid complexes. Thus, in the presence of the ionic detergent the protein remained in the complexes. Based on this finding, it was concluded that the minimal activity of the inhibitor

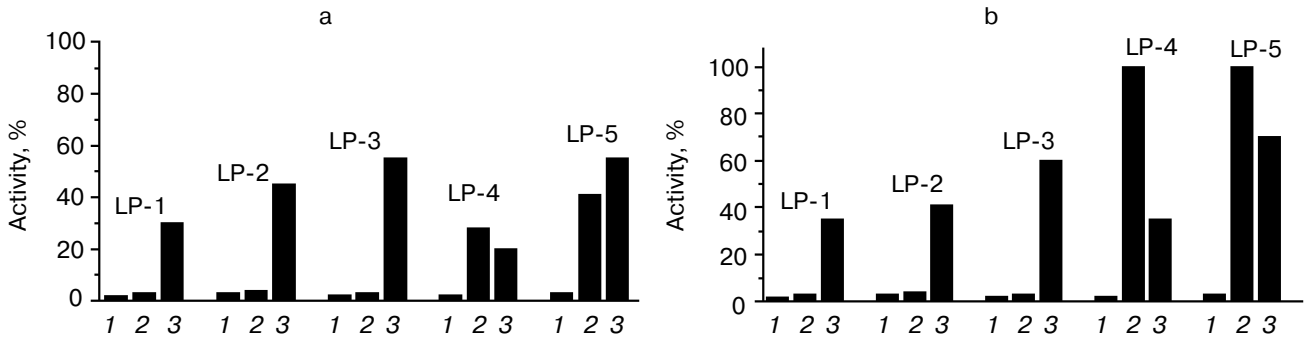

Fig. 2. Antitryptic activity of BPTI–lipid complexes precipitated at pH 3.0 (a) and 8.0 (b) after 1 h (*1*), after 1 week (*2*), and after 1 h in the presence of SDC (*3*).

in the complex was not associated with its irreversible inactivation but was probably caused by blocking of the active site of the inhibitor. However, the low activity of the inhibitor in the complex suggested that the protein is bound to the MLV in a region located near the active site. This hypothesis was confirmed by the finding that the inhibitor activity in the complex could be restored by addition of SDC, which converted the MLV into micelles [17]. In this state the active site of the inhibitor is accessible to trypsin.

On the other hand, for two preparations (LP-4 and LP-5) the inhibitor–lipid interaction was reversible. After storing the complex for one week at 0°C, pH 8.0, i.e., under conditions that retain BPTI activity, these preparations were found to regain up to 100% of the inhibiting activity (Fig. 2). Electrophoresis showed that free protein appeared under these conditions, i.e., the complex was destroyed. The complex size was determined by quasi-elastic light scattering; it was found that after storage of aqueous dispersions of LP-4 and LP-5 for a week the number of particles of more than 1 μm in size decreased from 60-70 to 20-30%. In the other samples (LP-1, LP-2, and LP-3) the size of the protein–lipid complexes did not change during storage, and the protein was entirely retained in the complexes, which shows that the complexes were stable. It seems that fragmentation of the protein–lipid complex particles resulted in release of native protein with high initial activity. Thus, this experiment, like the experiment with SDC, confirmed that complexing the protein with the lipids did not inactivate the protein.

Thus, in the present work the non-membrane water-soluble protein BPTI was shown to induce aggregation and precipitation of MLV, and the efficiency of this process was shown to depend on pH and the content of negatively charged components in the lipid preparations. It was concluded that the aggregation was mainly caused by electrostatic interaction between negative groups in the lipid molecules and positive groups of the protein. The activity of BPTI–MLV complexes was studied, and the inhibitor in the complex was found to display high activity under conditions similar to physiological ones in the duodenum (the presence of SDC, pH 8.0). It was shown by determination of activity and size distribution of the protein–lipid complexes during storage that the interaction was reversible and that the protein could dissociate from the complex in the case of low content of negatively charged components (LP-4, LP-5). In the case of high content of negatively charged components (LP-1, LP-2, and LP-3), the complex was stable during the storage.

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REFERENCES

1. Epand, R. M. (1998) *Biochim. Biophys. Acta*, **1376**, 353-368.
2. Brasseur, R., Pillot, T., Lins, L., Vandekerckhove, J., and Rosseneu, M. (1997) *Trends Biochem. Sci.*, **22**, 167-172.
3. Goldberg, E. H., and Zidovetzki, R. (1998) *Biochemistry*, **37**, 5623-5632.
4. Klyachko, N. L., Levashov, A. V., Kabanov, A. V., Khmelnitsky, Yu. L., and Martinek, K. (1991) in *Kinetics and Catalysis in Microheterogenous Systems. Surfactant Sci. Ser.* (Gratzel, M., and Kalyanasundaram, K., eds.) Vol. 38, Marcel Dekker, N. Y.-Basel, pp. 135-181.
5. Liu, D., and Huang, L. (1992) *Analyt. Biochem.*, **202**, 1-5.
6. De Arcuri, B. F., Vechetti, G. F., Chehin, R. N., Goni, F. M., and Morero, R. D. (1999) *Biochem. Biophys. Res. Commun.*, **262**, 586-590.
7. Folch, J., Lees, V., and Sloan-Stanley, G. F. (1957) *J. Biol. Chem.*, **226**, 497-503.
8. Martynova, O. M., Sorokoumova, G. M., Selishcheva, A. A., Alekseeva, S. G., Tiourina, O. P., and Shvets, V. I. (2000) *Byull. Eksp. Biol. Med.*, **129**, 159-162.
9. Klenin, V. I., Shchegalev, S. Yu., and Lavrushin, V. I. (1977) *Characteristic Functions of Disperse Systems* [in Russian], Saratov University Publishers, Saratov, p. 117.
10. Warner, G. (1983) *Colloid Polym. Sci.*, **261**, 508-519.
11. Ditmer, J. C., and Lester, R. L. (1964) *J. Lipid Res.*, **5**, 126-129.
12. Rodriguer-Vico, F., Martinez-Caynela, M., Garcia-Perigrin, E., and Ramirez, Y. (1989) *Analyt. Biochem.*, **183**, 275-278.
13. Kassel, B., Radicevic, M., Berlow, S., Peanasky, R. J., and Laskowski, M. S. (1963) *J. Biol. Chem.*, **238**, 3274-3278.
14. Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962) *Nature*, **195**, 281-290.
15. Wolf, W. J., and Thomas, B. W. (1971) *J. Chromatogr.*, **57**, 281-293.
16. Ivkov, V. G., and Berestovskii, G. N. (1982) *Lipid Bilayer of Biological Membranes* [in Russian], Nauka, Moscow, p. 223.
17. Selishcheva, A. A., Miroshnikova, T. A., Voronin, M. V., and Vasilenko, I. A. (1993) *Biochemistry* (*Moscow*), **53**, 202-207.