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Interaction of Native Bowman–Birk Soybean Protease Inhibitor and Its Hydrophobized Derivative with Multilamellar Vesicles of Soybean Phospholipids

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Abstract—The interaction of native Bowman–Birk soybean protease inhibitor (BBI) and its hydrophobized derivative with multilamellar vesicles of various soybean phospholipids was investigated. Decrease in pH and introduction of negatively charged components to the lipid mixture increased BBI content in the protein–lipid complex. This suggests a contribution of electrostatic forces in the protein–lipid interaction. Protein hydrophobization insignificantly influenced BBI binding to lipids. In the complex with lipids, both proteins (BBI and its hydrophobized derivative) retained high anti-chymotrypsin activity (75-100%), which was not influenced by the presence of the ionic detergent sodium deoxycholate.

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Key words: protein–lipid complexes, multilamellar vesicles, phospholipids, Bowman–Birk soybean protease inhibitor

Studies on protein–lipid interactions have been traditionally focused on integral or peripheral membrane proteins that were directly involved in regulation of functional activity of membranes [1]. Now study of interactions of non-membrane proteins with aqueous phospholipid dispersions has become more active due to practical application of protein–lipid complexes in pharmacology [2].

Non-membrane proteins are hydrophilic proteins that are not functionally linked to plasma or intracellular membranes; nevertheless these proteins may form complexes with phospholipids *in vitro* and possibly *in vivo*. Generally, binding energy for protein–lipid complex formation has two components: one component is responsible for electrostatic interaction, whereas the other one determines non-electrostatic interaction. The latter component originates from energy provided by various processes. These include transfer of protein segment from polar aqueous phase to less polar region of membrane surface formed by negatively charged phospholipid heads and transition of the protein molecule into the "molten globule" state [3] or change in structural organization of the phospholipids [4].

In this study we have analyzed the interaction of various soybean phospholipid preparations with a water-soluble non-membrane protein, Bowman–Birk soybean protease inhibitor (BBI), which exhibits anti-inflammatory, anti-carcinogenic, and anti-tumor activities [5]. For evaluation of the putative contribution of electrostatic and non-electrostatic components into phospholipid interaction with BBI, we have investigated the composition of complexes obtained for native BBI and its hydrophobized derivative with soybean lipids containing various amounts of negatively charged components. The hydrophobized derivative of BBI was obtained by introduction of two residues of oleic acid into the protein molecule [6]. We also investigated the dependence of anti-protease activity of resultant protein–lipid complexes on lipid composition.

Abbreviations: BBI) Bowman–Birk soybean protease inhibitor; BPTI) basic pancreatic trypsin inhibitor; DOC) sodium deoxycholate; LP) soybean lipid preparation; MLV) multilamellar vesicles; PC) phosphatidylcholine; PE) phosphatidylethanolamine; PG) phosphatidylglycerol; PI) phosphatidylinositol; lyso-PC) lysophosphatidylcholine.

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MATERIALS AND METHODS

The following reagents were used in the study: trypsin and chymotrypsin containing 64 and 57% of active molecules, respectively, and sodium deoxycholate (DOC) were from Merck (Germany); phosphatidylcholine (PC) and lyso-phosphatidylcholine (lyso-PC) of 98% purity were from Lipoid (Germany); the substrates ethyl ester of N-benzoyl-L-arginine (BAEE) and ethyl ester of N-benzoyl-L-tyrosine (BTEE) were from Sigma (USA); solvents were from Khimmed (Russia). BBI was isolated from soybean flour as described earlier [7, 8]. The content of active inhibitor in the purified preparation of BBI determined by titration of trypsin and chymotrypsin (with known content of active sites) was 79 ± 2%. Hydrophobized BBI ((oleoyl)$_2$-BBI) obtained by acylation of oleic acid derivative as described earlier [6] was kindly supplied by O. P. Tyurina. The content of active inhibitor molecules in (oleoyl)$_2$-BBI was 66 ± 3% (by mass). Soybean lipid extract, lipid preparation 1 (LP-1), was obtained using semi-skimmed soybean flour (State Standard (GOST) 3898-56; Assoya, Krasnodar) by the method of Folch et al. [9]. Methods for isolation of other lipid preparations and determination of their composition were described earlier [10].

Preparation of multilamellar vesicles (MLV) of phospholipids. Aqueous dispersions of lipid preparations were obtained as MLV by dispersing samples dried in 0.01 M HCl by the method of multiple freezing in liquid nitrogen and thawing.

Preparation of complexes of multilamellar vesicles with native and (oleoyl)$_2$-BBI. The complexes were prepared at pH 2.0. Aqueous dispersion of phospholipid preparations (5 mg/ml, 1 ml) was mixed with 0.05 ml of BBI preparations (10 mg/ml). Samples were incubated for 10 min. The precipitates formed were separated by centrifugation at 500*g* for 10 min and dispersed in 1 ml of 0.05 M Tris-HCl buffer, pH 8.0, for preparation of protein–lipid complexes. Purity of complexes was monitored by electrophoresis in 15% polyacrylamide gel using the system of Reisfeld et al. [11]. Protein content was determined by a modified Lowry method [12] and phospholipid content was determined by the method of Dittmer [13].

Determination of anti-trypsin activity of the protein–lipid complexes. After sequential addition of 0.1 ml of trypsin solution (0.1 mg/ml in 1 mM HCl) and an aliquot of diluted protein–lipid complex solution (15-30 µl) to a 1-ml cuvette, the final volume was adjusted to 0.8 ml with 0.05 M Tris-HCl buffer, pH 8.0. The mixture was incubated at room temperature for 7 min. The reaction was initiated by adding 0.2 ml of 1.5 mM BAEE solution in 0.05 M Tris-HCl buffer, pH 8.0. Changes in optical density were registered at 253 nm using a UV-265FW spectrophotometer (Shimadzu, Japan).

In parallel samples, rates of substrate hydrolysis catalyzed by trypsin were measured in the presence and in the absence of individual lipids for evaluation of the amount of phospholipid-bound trypsin.

Determination of anti-trypsin activity of protein–lipid complexes in the presence of DOC. Aqueous dispersion of protein–lipid complexes (0.02 ml) were mixed with equal volume (0.02 ml) of 5% DOC. Samples were incubated for 0.5 h, diluted, and then 15-30-µl aliquots were taken for determination of anti-trypsin activity of the inhibitor in the protein–lipid complexes. In parallel samples rates of substrate hydrolysis catalyzed by trypsin were measured in the presence and in the absence of individual lipids and DOC for evaluation of the amount phospholipid-bound trypsin and trypsin inactivated in the presence of DOC. The calculated amount of active inhibitor in the complexes in the presence of the detergent was corrected for detergent effect on the native protein.

Determination of anti-chymotrypsin activity of protein–lipid complexes. After sequential addition of 0.1 ml of chymotrypsin solution (0.05 mg/ml in 1 mM HCl) and an aliquot of diluted protein–lipid complex solution (15-30 µl) to a 1-ml cuvette, the final volume was adjusted to 0.9 ml with 0.05 M Tris-HCl buffer, pH 8.0. The mixture was incubated at room temperature for 7 min. The reaction was initiated by adding 0.1 ml of 1.27 mM BTEE solution in a mixture of 0.05 M Tris-HCl buffer, pH 8.0, and methanol (3 : 1 v/v). Changes in optical density were registered at 256 nm using the UV-265FW spectrophotometer.

In parallel samples, rates of substrate hydrolysis catalyzed by chymotrypsin were measured in the presence and in the absence of individual lipids for evaluation of the amount of phospholipid-bound chymotrypsin.

Determination of anti-chymotrypsin activity of protein–lipid complexes in the presence of DOC. Determination of the effect of DOC and its control were carried out under the same conditions as described above for experiments on determination of anti-trypsin activity.

RESULTS AND DISCUSSION

Selection of lipid preparations. Soybean lipid preparation (LP-1) was selected as the initial system for complex formation with BBI. This preparation contains phospholipids of various classes: negatively charged phospholipids, zwitterions, and lyso-components (Table 1). The phospholipid composition of LP-1 has been analyzed earlier [10]; it shares similarity with that of eukaryotic biomembranes. Besides phospholipids, LP-1 also contains fatty acids, saponins, and minor quantities of glycolipids, sterols, and proteins. Since LP-1 is a rather complex lipid preparation, it has been used for sequential purification of a series of lipid preparations (LP-2, LP-3, LP-4, LP-5) characterized by simpler composition. Purification procedures have been described earlier [10, 14] and Table 1 shows the composition of these prepara-

Table 1. Composition of lipid preparations

Preparation	Lipid content, % of preparation mass	Non-phospholipid contaminations, % of preparation mass			Content of individual phospholipids, % of sum of all phospholipids				
		fatty acids	saponins	proteins	PC	PE	PI	PG	lyso-PC
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	100	—	—	—	56 ± 2	20 ± 2	8 ± 1	8 ± 1	7 ± 1
LP-4	100	—	—	—	45 ± 2	22 ± 2	33 ± 2	—	—
LP-5	100	—	—	—	67 ± 2	33 ± 2	—	—	—

tions. Phospholipid preparations used in experiments differed by one or two components. This facilitated understanding of the putative role of each component in the interaction with proteins.

Complex formation between multilamellar vesicles and BBI. The complex formation was based on aggregation of phospholipid molecules during protein addition, resulting in sedimentation of protein–lipid complex [14]. This approach yielded a complex lacking native protein and protein-unbound phospholipids. Sedimentation of BBI with lipid preparations was observed at pH < p*I*, when the protein was positively charge. BBI is an acidic protein with p*I* ~ 4.5 [15]. Figure 1 shows that optimal sedimentation occurred at pH 2.0 (this is the lowest limit of pH stability of the protein). This suggests involvement of electrostatic interactions between positively charged protein and negatively charged MLV.

For optimization of conditions of complex formation, we investigated dependence of BBI content in the precipitate on phospholipid concentration. Figure 2 shows that the maximal protein content in the precipitate was found at phospholipid concentration of 5 mg/ml. (We mean net concentration of phospholipids of the lipid preparations used.) This concentration was used in subsequent experiments.

Electrophoresis in 15% polyacrylamide gel [11] revealed lack of a protein band corresponding to the native inhibitor and the presence of a band corresponding to complex of higher molecular mass exhibiting lower electrophoretic mobility. This suggests that dispersion of the precipitate of BBI–lipid complexes in Tris-HCl buffer, pH 8.0, was not accompanied by their decomposition.

Table 2 shows results of analysis of BBI–lipid complexes. In all preparations the complex involved more than 60% of the phospholipids. Maximal amounts of phospholipid and protein were found in BBI–LP-1 complex. Lack of saponins in the lipid preparation (LP-2) reduced the mass portion of phospholipids in the complex to 77%. In the case of the lipid preparations containing only the phospholipids lyso-PC, phosphatidylglycerol (PG), and phosphatidylinositol (PI), 83% of the phospholipids were found in the complex. The minimal amount of phospholipids was bound to BBI when the lipid preparation contained phospholipid zwitterions (LP-5).

Similar dependence was also found for the protein component: the amount of BBI bound to MLV in the water-insoluble complex at pH 2.0 decreased with decrease in content of negatively charged non-phospho-

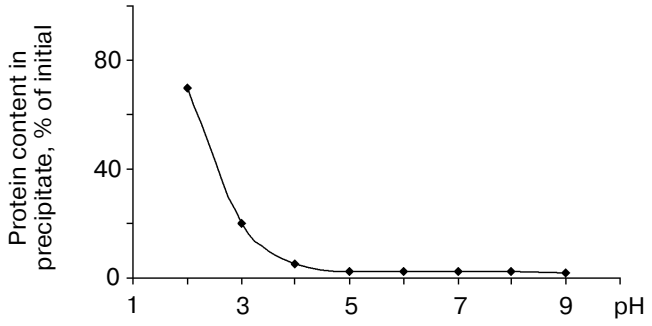

Fig. 1. Effect of pH on protein content in the precipitated complex BBI/LP-2.

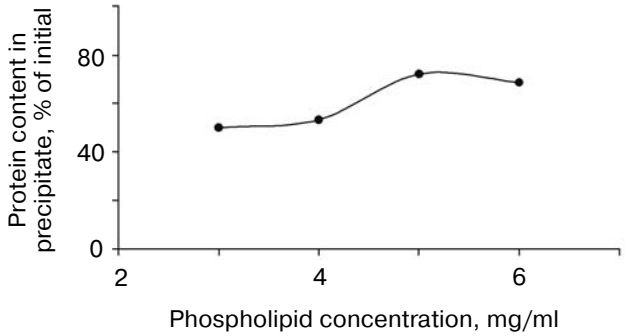

Fig. 2. Dependence of BBI content in BBI/LP-2 complex on phospholipid concentration at pH 2.0.

Table 2. Comparative characteristics of protein–lipid complexes

Component of protein–lipid complex	Precipitated in the complex, % of initial	Component of protein–lipid complex	Precipitated in the complex, % of initial
LP-1	90 ± 2	BBI	73 ± 2
LP-1	94 ± 2	$(oleoyl)_2$-BBI	71 ± 3
LP-2	77 ± 3	BBI	63 ± 3
LP-3	83 ± 2	BBI	49 ± 2
LP-4	80 ± 2	BBI	40 ± 2
LP-5	65 ± 2	BBI	30 ± 1

lipid and phospholipid components (fatty acids, PI, PG) in lipid preparations. This is consistent with the modern viewpoint on the electrostatic nature of protein binding to MLV. Earlier studying interactions of lipid preparations with basic pancreatic trypsin inhibitor (BPTI), p*I* 10.5 [14], and also trypsin, p*I* 10.5 [16], we came to the same conclusion.

Anti-protease activity of BBI in the complex with multilamellar vesicles. Anti-trypsin activity of BBI complexes with MLV is rather low (20-40%) (Fig. 3a). Minimal activity was typical for protein complexes with LP-2 and LP-5 (~20%), two-times higher activity was detected in the case of protein complexes with LP-1, LP-3, and LP-4 (~40%). Higher anti-protease activity may be attributed to the presence of significant amounts of lyso- and negatively charged lipid components.

According to ^{1}H-NMR data on the 3D-structure of BBI in solution [17], the trypsin-binding domain of BBI includes amino acid residues 7-26 and 54-63. Relatively low anti-trypsin activity of MLV complexes with native BBI may be explained by electrostatic interaction between negatively charged polar heads of phospholipids with two positively changed amino groups (of six present in the BBI molecule), particularly Lys16 (included in the so-called trypsin-reactive BBI center) and Lys63.

Studying properties of protein–lipid complexes of trypsin and BPTI with the same set of phospholipid preparations (LP-1-LP-5), we demonstrated that activity of the resulting complexes was significantly lower than in native proteins; the activity depended on precipitating pH value and content of negatively charged components in the lipid preparations [14, 16]. In those complexes inhibitory activity of BPTI did not exceed 5% and esterase activity of trypsin was ~42%. In the latter case, we found nontrivial dependence of catalytic activity on complex concentration.

Significant reduction in trypsin activity and anti-trypsin activities of two protein inhibitors suggests that protein–lipid complex formation results in blockade of protein active sites by phospholipid aggregates. For evaluation of this hypothesis, we investigated the effect of sodium deoxycholate on anti-trypsin activity of BBI complexes with lipids. The presence of DOC caused 2-2.5-fold increase in anti-trypsin activity of LP-1, LP-2, and LP-4 protein–lipid complexes and did not influence anti-trypsin activity of LP-3 and LP-5 complexes (Fig. 3a). Insignificant increase in anti-trypsin activity of protein–LP-5 complex by DOC may be attributed to the fact that LP-5 contains only phospholipid zwitterions. Lower activity of BBI complex with LP-3 in the presence of DOC than in corresponding complex with LP-4 is determined by the presence of PI carrying two negative charges. Earlier we demonstrated that DOC increased anti-trypsin activity of BPTI during its complex formation with LP-1-LP-5 by more than 3-fold [14].

Dynamic light scattering revealed that DOC decreased sizes of aggregates of BBI with LP-1 and LP-2 by one order of magnitude (from 1.33 to 0.11 μm and from 1.43 to 0.13 μm for BBI/LP-1 and BBI/LP-2 complexes, respectively). It is possible that during this process anti-trypsin active site of these inhibitors becomes more susceptible to the enzyme. These data are in agreement with results obtained in [18], where it was demonstrated that during the increase in lipid/detergent molar ratio the size of PC aggregate/detergent reduced to 50 nm.

Complex formation between BBI and MLV insignificantly decreased its anti-trypsin activity (Fig. 3b). Anti-chymotrypsin activity was minimal during complex formation with LP-4 (~76%) and maximal during protein binding to LP-2 (~100%). 2D-^{1}H-NMR spectroscopy revealed [17] that the chymotrypsin-binding domain of BBI includes residues 27-53. Higher anti-chymotrypsin activity of BBI complexes with MLV compared with their anti-trypsin activity may be explained by the behavior of the side chain of Lys37 of the chymotrypsin-binding domain, which has to be fully exposed to solution for interaction with phospholipids. So the chymotrypsin-reactive site Leu43–Ser44 is insignificantly shielded.

In the presence of DOC (Fig. 3b) the complexes retained high (≥75%) anti-chymotrypsin activity. It should be noted that anti-trypsin and anti-chymotrypsin

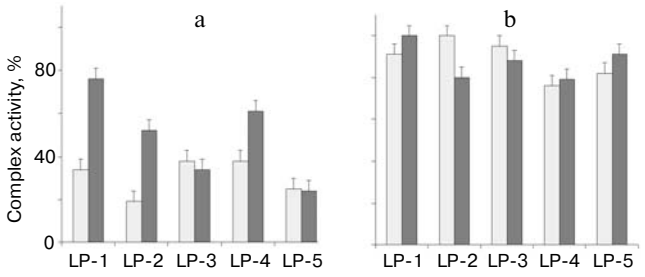

Fig. 3. Anti-trypsin (a) and anti-chymotrypsin (b) activities of BBI–lipid complexes in the absence (light columns) and in the presence (dark columns) of DOC.

activities of BBI–lipid complexes were maximal with LP-1 the presence of DOC. For the complexes of the same lipid preparations with BPTI it was demonstrated that in the presence of DOC, the anti-trypsin activity was maximal for the system with LP-5 in the case of complex formation at pH 8.0, and for the system with LP-3 in the case of complex formation at pH 3.0 [14]. This suggests that binding of phospholipids with water-soluble protein depends not only on properties of the lipid preparations, but also on the protein nature.

Comparison of interaction of native and hydrophobized BBI with phospholipids. For evaluation of the role of hydrophobic interactions in formation of BBI–lipid complexes, we compared some characteristics of protein–lipid complexes (protein content in precipitate, its activity, etc.) obtained with native and hydrophobized protein under the same conditions. The protein was modified using oleic acid derivatives by two of five acylation-susceptible amino groups in the BBI molecule [6]. Modification of each of these five groups was equally possible. So in the resultant preparations containing three free amino groups susceptible for titration two modified groups may have various positions; for example, both groups may be positioned in the central part of molecule or near the anti-trypsin center [17]. For studies of complex formation with the acylated derivative of BBI we selected LP-1, because protein–lipid complex containing native BBI and LP-1 was characterized by high yield and maintenance of inhibitory activity (Table 2).

Data of Table 2 show that modification of the inhibitor with two oleoyl residues caused insignificant increase in protein content in the precipitate.

Orientation of active sites of the inhibitor versus the lipid bilayer and the putative role of hydrophobic interactions were evaluated by comparison of activity of protein–lipid complexes containing native and acylated BBI preparations. Since $(oleoyl)_2$-BBI had somewhat lower anti-protease activity (by 13 ± 2%) than native inhibitor, their activities within complexes with MLV were calculated versus the corresponding lipid-unbound BBI preparation. This approach allows detecting altered activity as a result of vesicle interaction with acylated inhibitor molecules.

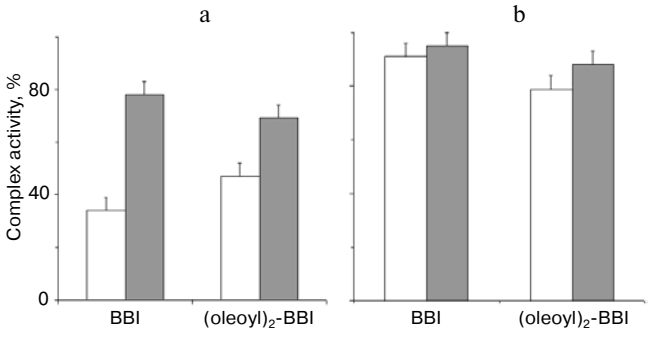

Fig. 4. Anti-trypsin (a) and anti-chymotrypsin (b) activities of LP-1 multilamellar vesicle complexes with native and $(oleoyl)_2$-BBI in the absence (light columns) and in the presence (dark columns) of DOC.

Differences between anti-protease activities of $(oleoyl)_2$-BBI–MLV complex and the corresponding native BBI–MLV complex did not exceed 15% (Fig. 4). This suggests possible involvement of oleoyl residues located in the central part of the protein molecule into the interaction with the lipid bilayer. This increases susceptibility of the anti-trypsin site and shielding of the anti-chymotrypsin site.

Addition of DOC increased anti-protease activity of both protein–lipid complexes. Interaction with DOC (Fig. 4b) was accompanied by a slight increase in anti-chymotrypsin activity of BBI complexes and the activity of $(oleoyl)_2$-BBI complexes increased by 9%. This suggests that addition of ionic detergent may regulate activity of not only BBI–lipid complexes, but also activity of complexes containing MLV and hydrophobized vesicles. In the presence of DOC, anti-trypsin activity (Fig. 4a) was higher in protein–lipid complexes containing native protein than in the complexes containing the hydrophobized inhibitor. The latter may be due to additional hydrophobic interactions between oleoyl residues (of the hydrophobized protein) with phospholipids.

Thus, a series of studies on the interaction of MLV obtained from soybean lipids and three water-soluble proteins (BBI, BPTI [14], and trypsin [16]) differing in their p*I* and structure revealed that electrostatic forces are the primary factor determining aggregation of protein–lipid complexes. Subsequent orientation of the protein globule involves hydrophobic interaction leading to stabilization of protein–lipid complexes. The coincidence of major principles underlying MLV interactions with BPTI and with trypsin is consistent with identical p*I* values of these proteins. However, there are significant differences in composition of lipid complexes with BPTI and trypsin. These include phospholipid/protein molar ratios. With the exception of LP-1 (pH 8.0) the lipid binding capacity of trypsin was 1.5-2.5 times higher than that of BPTI. This may reflect: 1) different molecular mass and size of these proteins; 2) different secondary structure of these proteins (BPTI has two α-helices and one β-sheet [PDB, 1PIT], whereas trypsin has four α-helices and two β-sheets [PDB, 1EB2]; 3) different ability for hydrophobic interactions (originating from the abovementioned differences). According to data from [19], hydrophobic interactions appear during insertion of elements of secondary structure (α-helices and β-sheet) into lipid bilayer.

BBI lacks α-helices and contains a sequence of two β-sheets [PDB 2BBI]. It has the following characteristic structural features [17, 20]: lack of hydrophobic core, typical for water-soluble proteins including low molecu-

lar weight protease inhibitors of other families; existence of two hydrophobic solvent susceptible regions; seven disulfide bonds apparently prevent collapse of these hydrophobic regions and formation of the hydrophobic core. These features emphasize the intermediate position of BBI between typical water-soluble proteins and peripheral membrane proteins. The mode of interaction of BBI with MLV lipid bilayer is very similar to that of peripheral membrane proteins such as myelin basic protein [21], cytochrome *c* [22], water-soluble myelin apolipoprotein [23], apolipophorin III [24], etc. In these cases stronger interaction was observed with negatively charged phospholipids and at pH < p*I*. This indicates electrostatic nature of interactions.

The results of the present study may have important applications. For example, phospholipids may be considered as a biodegradable matrix, which can gradually release drugs and protein–lipid complexes and might be recommended for per oral use. It is important to stress that DOC concentration is close to its physiological concentration in duodenal juice. Consequently, study of DOC effect on activity of protein–lipid complexes can mimic their behavior in the small intestine. Thus, the following behavior of protein–lipid complexes in the gastrointestinal tract could be proposed. Since these complexes are dispersed at alkaline pH values, they pass the stomach in their intact state. In the small intestine where the pH is about 8.0, the protein–lipid complexes will be gradually dispersed and in the presence of ionic detergents they will regain biological activity. So, we conclude that protein–lipid complexes are promising drug composites for per oral use.

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