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INTERACTION OF LIPID-TRANSPORT PROTEINS FROM *Nigella sativa* SEEDS WITH LIPID MEMBRANES

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Three pure lipid-transport proteins (LTPs) were isolated from Nigella sativa *seeds. The effects of the three LTPs on release of ANTS probe from the inner volume were studied using fluorescence in liposomes of different lipid composition. It was shown that all LTPs had a dose-dependent effect on the liposome permeability.*

Keywords: *Nigella sativa*, seeds, lipid-transport proteins, phospholipids, fluorescent probes, fluorescence quenchers.

Non-specific lipid-transport proteins (LTPs) belong to a family of proteins that are widely distributed in plants [1, 2]. They were isolated from seeds of various classes of plants such as maize [3], wheat [4], barley [5], and rice [6] in addition to leaves of several plants [7]. A comparison of the physicochemical properties of the isolated compounds reveals common trends in the molecular structure. They are a homogeneous family of basic peptides 90–93 amino acids long with eight cysteine units in conserved positions that form a hydrophobic cavity [8]. LTPs are typically capable *in vitro* of transferring glycerophospholipids or galactolipids through membranes [1, 8]. They can inhibit *in vivo* the growth of bacteria and fungi [8]. One of the proposed mechanisms of the antifungal activity of several LTPs (Ha-AP10 from *Helianthus annuus* [9], LTP2 from wheat [10], and LTP from onion [11]) is the induction by them of increased permeability of pathogen plasmatic membranes for small organic molecules and inorganic ions.

It would be useful to find trends in their effects on the structural properties of lipid membranes in order to understand better differences in the effectiveness with which LTP from various sources interact with lipids.

Our goal was to study the effects of three LTP isolated from *Nigella sativa* seeds (Ns-LTPs) that had different physicochemical properties (LTP1, LTP2, LTP3) on the permeability of model lipid membranes.

We have previously isolated and studied the physicochemical properties of Ns-LTP1 from *N. sativa* seeds [12]. Here we continued the detailed investigation of the peptide composition from *N. sativa* seeds and found two more peptides that belonged to the same class as LTP1 but had different molecular weights (MWs).

Chromatography of total peptides from *N. sativa* seeds over affinity sorbent Hi Trap Heparin HP produced four basic fractions that were eluted by different NaCl concentrations (0 M, 0.1, 0.5, and 1). According to mass spectrometry, a component with MW 8130 Da dominated the fraction eluted by buffer A. Then, the component was purified by RP-HPLC, which produced four fractions. Mass spectral analysis of these showed that fraction 4 was homogeneous and contained a polypeptide of MW 8130 Da (LTP2).

The 0.1-M fraction contained several protein components with MW in the range 9–12 kDa. The dominant polypeptide had MW 9602 Da. The polypeptide was purified by RP-HPLC, which produced eight fractions. Mass spectral analysis of these showed that fraction 4 was homogeneous and contained a component with MW 9602 Da, which we called Ns-LTP1. We sequenced its *N*-terminus and determined the amino-acid composition and number of cysteines in order to confirm that it was an LTP [12].

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TABLE 1. Release of ANTS Probe from Liposomes upon Addition of Three LTPs Isolated from *Nigella sativa* Seeds to Liposomes Formed of PC and 90% PC + 10% DMPS (Mixture)

LTP type	ANTS release from liposomes, %*						
	C_{LTP}/C_{PC}			C_{LTP}/C_{mix}			
	0.02	0.04	1	0.04	0.1	0.2	1
LTP1	0	0	33	0	0	0	56
LTP2	12	22	36.6	0	14.6	26	80
LTP3	0	0	22	0	6	11	51

*Uncertainty of ANTS release from liposomes was <5% for series of three measurements.

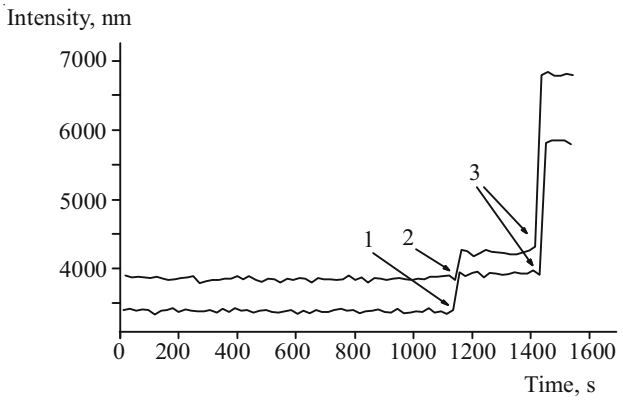

Fig. 1. ANTS fluorescence kinetic curves upon addition of LTP2 to liposomes containing ANTS + DPX for C_{LTP}/C_{lip} 1:25 and 1:50: addition of LTP2 (1:25) (1), addition of LTP2 (1:50) (2), addition of Triton X-100 (3).

A component with MW 9703 Da dominated the 0.5-M fraction. It was purified by RP-HPLC, which produced two fractions. Mass spectral analysis showed that fraction 1 was homogeneous and contained a component with MW 9703 Da (LTP3).

Liposomes loaded with the fluorescent probe 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) (dye family) and a quencher of ANTS fluorescence, *p*-xylene-*bis*-pyridinium bromide (DPX), were formed in order to study the effects of Ns-LTPs on the permeability of model lipid membranes.

We used two types of liposomes prepared from phosphatidylcholine (PC) and its mixture with dimyristoylphosphatidylserine (DMPS) (90% PC + 10% DMPS) that contained in the inner volume ANTS and DPX in order to study the effects on membranes of LTP1, LTP2, and LTP3 isolated from *N. sativa* seeds. The percent content of anionic phospholipid DMPS corresponded approximately to its natural content in cellular structures.

The experimental results showed that LTP1, LTP2, and LTP3 were capable of inducing release of ANTS and DPX from the PC liposome inner volume. Figure 1 shows an example of the experimental kinetic data for the change of ANTS fluorescence intensity upon adding Ns-LTP2 in various ratios of its concentration to the PC concentration (C_{LTP}/C_{lip}). The percent release of ANTS from the liposome inner volume was calculated relative to the probe fluorescence intensity after adding the detergent Triton X-100, for which the ANTS release was 100%. Table 1 presents experimental results for all three LTPs.

LTP1 and LTP3 induced release of the fluorescent probe at a high C_{LTP2}–C_{lip} ratio (1:1). Therefore, Ns-LTP2 had higher affinity for PC molecules than the other two proteins.

The percent release of ANTS from the inner volume showed a dose-dependent increase upon adding the three tested Ns-LTPs to the negatively charged liposomes formed from the mixture (90% PC + 10% DMPS) when compared with liposomes prepared from pure PC. Table 1 summarizes the experimental results. A comparison of the percent release of ANTS from the liposomes shows that the effect was greatest upon adding Ns-LTP2; intermediate, for Ns-LTP3; and least, for Ns-LTP1 with $C_{LTP}/C_{lip} < 1$.

Thus, it was confirmed unambiguously that the release of ANTS from the inner volume of liposomes formed from pure PC or from a PC + DMPS mixture depended on the ratio C_{LTP}–C_{lip} upon adding to it LTP1, LTP2, and LTP3. The effect was dose-dependent. Ns-LTP2 of all tested LTPs from *N. sativa* seeds had a more pronounced ability than LTP1 and LTP3 to induce release of ANTS from the PC liposome inner volume and especially from negatively charged liposomes formed from 90% PC + 10% DMPS. This agreed with the literature [13]. This also confirmed the conclusion that the presence of anionic lipids in model and biological membranes increases the probability of forming pores or defects through which small organic molecules and inorganic ions can flow. In certain instances, this can explain the fungal toxicity of Ns-LTP isolated from various sources [8-11]. It can be hypothesized that the difference in the effectiveness for forming pores or inducing defects in membrane lipid bilayers may be related to structural features of the isolated LTP.

EXPERIMENTAL

Acidic Extraction of Peptides and Proteins. Previously ground and de-fatted *N. sativa* seeds were extracted with acetic acid (10%) in de-ionized water using 10 mL of solution for 1 g of biological material (10 mL) in the presence of protease inhibitors (serine proteases trypsin, chymotrypsin, plasmin, callicrein, thrombin; cysteine proteases calpain, papain, catepsin B, catepsin L; aminopeptidases leucine aminopeptidase, alanyl aminopeptidase; serine and cysteine peptidases plasmin, trypsin, papain, catepsin B; acidic proteases pepsin, renin, catepsin D; and metalloprotease). For this, a mixture of protease inhibitors (30 μL) was added beforehand to an acid solution (10%) using 1 mL of inhibitors for 100 mL of extract obtained from 30 g of material. The extraction was carried out for 1 h at room temperature and constant stirring on a magnetic stirrer. The homogenate was centrifuged at 12,000 rpm. The precipitate was discarded. The supernatant was treated with acetone (1:5 ratio) and left overnight at 4°C. The acetone was decanted. The precipitate was dried in air and dissolved in TFA (0.1%). Insoluble compounds were removed by centrifugation.

Desalting of the Sample. The dissolved precipitate was desalted by reversed-phase high-performance liquid chromatography over an Aquapore RP-300 C8 column (10 × 100 mm, GE Healthcare, USA) with elution at 1.5 mL/min at 38°C. The required eluent concentration was made up using two solutions, TFA (0.1%, solvent A) and TFA (0.1% containing 80% CH_3CN, solvent B). Detection used wavelength 214 nm. The column was equilibrated with solvent A before injecting the sample. Salts and other unbound components of the extract were eluted from the column by this same solvent. The protein-peptide fraction was desorbed from the column by 70% solvent B. The collected fraction was evaporated to dryness in a Speedvac vacuum concentrator (Savant, USA) and redissolved in Tris-HCl (10 mM, pH 7.2).

Affinity Chromatography. The desalted extract was separated by affinity chromatography using a stepped NaCl gradient over a Hi Trap Heparin HP column (2.5 × 5 cm) (Amersham Biosciences, England) that was equilibrated with Tris-HCl (10 mM, pH 7.2, buffer A). After eluting unbound components, proteins and peptides were eluted by a stepped NaCl gradient (0.1–0.5–1 M) in Tris-HCl (10 mM, pH 7.2) over 2 h at flow rate 1.2 mL/min at room temperature. Proteins and peptides were determined at 214 nm.

The collected fractions were evaporated to dryness in a Speedvac vacuum concentrator (Savant, USA) and redissolved in TFA (0.1%).

RP-HPLC over a Reprosile Pur ODS-300 C_{18} Column (4.6 × 250 mm). Components were purified by RP-HPLC over a Vydac C_{18} column (4.6 × 250 mm, Pharmacia Fine Chemicals, Sweden) equilibrated with 5% CH_3CN in TFA (0.1%). Elution was carried out for 60 min at flow rate 0.75 mL/min with a linear gradient from 5 to 40% CH_3CN in TFA (0.1%). Proteins and peptides were determined at 214 nm.

MALDI Mass Spectrometry. MWs of proteins and peptides were measured in an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) in positive-ion mode. The matrix was 2,5-dihydroxybenzoic acid. Mass spectra were processed using the Bruker DataAnalysis program. The accuracy of the measurements was 0.015%.

Preparation of Giant Liposomes Containing ANTS/DPX. We used the following phospholipids: egg phosphatidylcholine (PC), dimyristoylphosphatidylserine (DMPS) (Sigma, USA), fluorescent probe ANTS and its quencher DPX (Sigma).

Giant liposomes were prepared by a slightly modified literature method [14]. PC or PC/DMPS liposomes were prepared in aqueous solution containing HEPES (60 mM, pH 7.4), ANTS (25 mM), and DPX (90 mM). ANTS and DPX not

encapsulated by liposomes were removed over a Hi Trap TM column loaded with HEPES buffer (60 mM, pH 7.4). The eluent was diluted to lipid concentration up to 100 mM. ANTS fluorescence intensity was recorded at 25°C for excitation wavelength 353 nm and emission wavelength 520 nm. Complete (100%) release of ANTS from the liposome inner volume was observed upon addition of detergent Triton X-100 (2.5%, v/v) to the lipid samples.

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