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Effect of membranotropic and mucoadhesive formulations of protein proteinase inhibitors on bovine herpes virus-1 reproduction

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Abstract

The lipidized derivatives of Bowman–Birk soybean protease inhibitor (BBI) containing one to three oleoyl groups were synthesized and characterized. The $(ole)_1$- and $(ole)_2$BBI were demonstrated to have 200- and 100-fold higher uptake into Caco-2 cell monolayers compared to native BBI. The acylated BBI had increased affinity to elastase-like proteases. Aprotinin-loaded starch/bovine serum albumin microcapsules were prepared using interfacial cross-linking with terephthaloyl chloride and characterized for their morphology, size and release of the inhibitor. Various formulations of protein proteinase inhibitors were tested for their influence on BHV-1 reproduction in cell cultures. Native aprotinin possessed palpable dose-dependent effect inhibiting the virus reproduction up to 4.0 lg (10,000-fold). The bioadhesive, biodegradable aprotinin-loaded microcapsules were the most effective decreasing virus infectious titer up to 4.0 lg and delaying the cytopathic effect up to 144 h in lesser doses of aprotinin. The lipophilic derivative $(ole)_1$BBI was shown to exhibit effective inhibition (>100-fold) of BHV-1 reproduction unlike native BBI.

Keywords: Protease inhibitors; Lipidization; Microcapsules; BHV-1

1. Introduction

In the field of controlled release veterinary drug delivery, the utilization of new routes of administration or the development of new delivery systems are of considerable interest (Rathbone and Gurny, 2000).

The viral and bacterial respiratory–intestinal infections of cattle are the major causes of the diseases and death of the young cattle (Surin et al., 1991). Therefore, the complex prophylaxis and therapy of these infections are of great importance. Bovine herpes virus-1 (BHV-1) belongs to the subfamily α-herpesvirinae and is similar in structure to the other herpes viruses (Misra et al., 1981). The proteolytic processing appears to be an essential process in herpes virus growth, required for the formation of infectious virions (Gao et al., 1994).

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The BHV-1 maturational proteinase was shown to have Ser and His in its active site (Haanes et al., 1995). We supposed that the inhibitors of serine proteinases could interfere with BHV-1 reproduction. In order to verificate our assumption we decided to examine aprotinin and Bowman–Birk soybean protease inhibitor (BBI) as potential antiviral drugs. Besides our choice was supported with data that aprotinin has been found to inhibit the influenza virus reproduction in cell cultures, chicken embryos (Zhirnov et al., 1994) and to produce a therapeutic effect on influenza in animals (Zhirnov et al., 1982).

The antiviral drug nasal administration seems to be the most effective in infectious rhinotracheitis treatment since the upper airway serves as a "gateway to infection."

Recently, the novel approaches to nasal delivery of peptides and proteins were reviewed by Tirucherai et al. (2002). The following general guidelines were recommended to achieve effective nasal delivery: minimizing metabolism, prolonging residence time in the nasal cavity and increasing absorption (Tirucherai et al., 2002). Starch microspheres have been successfully used as bioadhesive nasal drug delivery systems characterized with raising the clearance time from nasal cavity to 3 h (Illum, 1999). The bioavailability of proteins in such formulations has been improved in various animal models (Illum, 1999).

Derivatization of peptides to produce compounds with improved lipophilicity is a promising approach for enhancing transport across the nasal mucosa (Tirucherai et al., 2002). Covalent attachment of fatty acids to polypeptides is now a widely recognized form of their modification. Fatty acids have been conjugated with such peptides/polypeptides and proteins as thyrotropin-releasing hormone (lauroyl-) (Muranishi et al., 1991); desmopressin (palmitoyl-) (Wang et al., 1999); insulin (palmitoyl-) (Hashimoto et al., 1989; Kurtzhals et al., 1995); aprotinin (oleoyl-, stearoyl-) (Tyurina et al., 1998); BBI (palmitoyl-) (Ekrami et al., 1995) (unsaturated fatty acids derivatives) (Malykh et al., 2001); α-chymotrypsin (stearoyl-) (Kozlova et al., 1999); superoxide dismutase (lauroyl-, caproyl-) (Ando et al., 1988); antibodies (stearoyl-) (Kabanov et al., 1990; Melik-Nubarov et al., 1993), etc.

Lipidization of peptides and proteins has been shown to enhance stabilization against proteolytic degradation (Bundgaard and Møss, 1990; Wang and Shen, 2000), binding to lipid membranes (Ando et al., 1988; Kamyshny et al., 1999; Kozlova et al., 1999) and transport cross-cell membrane (Chekhonin et al., 1995; Ekrami et al., 1995; Bundgaard and Møss, 1990), and prolong plasma half-life (Kurtzhals et al., 1995; Honeycutt et al., 1996; Wang et al., 1999).

There are a number of results, which demonstrate that lipidization is an effective approach for improving therapeutic efficacy of many polypeptide drugs (Kabanov et al., 1990; Kurtzhals et al., 1995; Honeycutt et al., 1996; Wang et al., 1999). On the other hand, lipidization may lead to the decrease of the biological activity of polypeptides (Hashimoto et al., 1989; Muranishi et al., 1991).

The objective of the present study was to reveal and to evaluate the effect of aprotinin both native and encapsulated in starch/bovine serum albumin microcapsules, as well as BBI both native and oleic acid-conjugated on the reproduction of BHV-1 in cell culture.

2. Materials and methods

2.1. Materials

Hydroxysuccinimide ester of oleic acid, citraconic anhydride, *N*-benzoyl-L-arginine-*p*-nitroanilide, MeOSuc(L-Ala)$_2$ProVal-*p*-nitroanilide and dimethyl sulfoxide were obtained from Sigma (USA). *N*-Benzoyl-L-tyrosine ethyl ester was purchased from Merck (Germany). Soluble starch (Glucidex 2) was supplied by Roqette Freres (France). Terephthaloyl chloride was from Aldrich–Chimie (France). Surfactants used were sorbitant trioleate (Span 80) and polyoxyethylene trioleate (Tween 85) from ICI (Germany). Chloroform, cyclohexane, dioxane and ethanol were from Prolabo (France). Aprotinin preparations (Gordox®, and Contrycal®) were purchased from Gedeon Richter (Hungary) and Arzneimittelwerk Dresden GmbH (Germany), respectively.

BBI, bovine serum albumin (BSA), bovine trypsin with 50% active protein content (Chase and Shaw, 1967) and α-amylase (27 U/mg) were obtained from Sigma (USA). α-Chymotrypsin with 57% active protein content (Shonbaum et al., 1961) was purchased from Merck (Germany). Human leukocyte elastase (HLE) with active enzyme molecule content 60% was

isolated as described earlier (Tikhonova et al., 1993). Fetal bovine serum was purchased from Life Technologies (Grand Island, NY).

The characterized embryonic bovine lung cell culture (EBL) and embryonic bovine kidney cell culture Taurus-1 (T-1) were from Veterinary Virology Department of Scryabin's Moscow State Academy of Veterinary Medicine and Biotechnology; BHV-1, strain "4016," was obtained from virus collection of Veterinary Virology Department of Scryabin's Moscow State Academy of Veterinary Medicine and Biotechnology.

2.2. Synthesis of BBI conjugates with oleic acid and their characterization

Partially citraconylated BBI was acylated by equimolar to remained NH_2-groups amount of *N*-hydroxysuccinimide ester of oleic (ole-) acid in a mixture of the organic solvents (dimethylsulfoxide–dioxane–pyridine) using a previously optimized procedure (Malykh et al., 2001). After vacuum evaporation of the solvents the protective moiety was removed at acidic pH and then the acylated derivatives of BBI were separated from the native protein by acid precipitation. The final yield of the conjugates was from 45% for $(ole)_1$BBI to 70% for $(ole)_3$BBI. Amino group contents in both the native and modified inhibitor preparations were measured spectrophotometrically using 2,4,6-trinitrobenzenesulfonic acid (Fields, 1971).

Electrophoresis of BBI preparations was carried out in 7% native polyacrylamide gel at pH 4.5 (Reisfeld et al., 1962).

The phase distribution of BBI preparations between Triton X-114 and 0.01 M Tris–HCl buffer pH 7.4, containing 0.15 M NaCl, was followed by protein determination (using Lowry's routine) in each phase (Bordier, 1981). The partition coefficients *P* (concentration in Triton X-114/concentration in aqueous phase) were calculated.

2.3. Preparation and characterization of aprotinin-loaded microcapsules

Microcapsules were prepared from soluble starch and BSA by interfacial cross-linking with terephthaloyl chloride at pH 9.8 as described elsewhere (Larionova et al., 1999a). Aprotinin-loaded microcapsules were prepared by incorporation of the inhibitor (0.4 and 0.8%) in the aqueous phase during the encapsulation (Larionova et al., 1999a). In order to sterilize microcapsules, γ-irradiation was used in the dose 15 kGy. Microcapsule morphology was studied by optical microscopy and scanning electron microscopy. Microcapsules were sized using a Coulter Multisizer II, Sampling Stand II A (UK).

2.4. In vitro aprotinin release studies

Sterilized lyophilized microcapsules (10 mg) were rehydrated in a test tube with 5 ml of PBS buffer (pH 7.4), containing α-amylase 0.2 mg/ml. The tube was incubated at 37 °C with constant agitation at 40 rpm. At appropriate time intervals, the sample was centrifuged and a 400 μl aliquot of the supernatant was withdrawn for protein content and antiprotease activity determination. 400 μl of fresh enzymatic solution was replaced to maintain sink conditions. Studies were repeated three times to determine represented means (±S.D.).

2.5. Determination of proteinase inhibition

The antitrypsin activity was determined from the decrease of the amidase activity of bovine trypsin using benzoyl-L-arginine-*p*-nitroanilide as the substrate (Kakade et al., 1969). The antichymotrypsin activity was determined from the decrease of the esterase activity of bovine α-chymotrypsin using *N*-benzoyl-L-tyrosine ethyl ester as the substrate (Hummel, 1959).

2.6. Determination of inhibition constants (K_i)

Inhibition constants of human leukocyte elastase (HLE) were determined by the method offered for slow, tight-binding inhibitors (Morrison and Walsh, 1988) using MeOSuc(L-Ala)$_2$ProVal-*p*-nitroanilide as a substrate (Stein et al., 1987).

2.7. The uptake of BBI derivatives by Caco-2 cells

Both native BBI and its conjugates were iodinated with ^{125}I by the chloramine-T method (Mc Conahey and Dixon, 1980). Caco-2 cells were grown on 6-well

cluster plates in DMEM supplemented with 10% fetal bovine serum. Confluent cell monolayers were treated with ^{125}I-BBI or ^{125}I-BBI-fatty acid derivatives in serum-free medium for 24 h (37 °C), and washed extensively using ice-cold PBS. Cell monolayers were trypsinized, detached, and centrifuged, and cell pellets were washed three times with cold PBS and counted in a gamma counter. Subsequently, cell pellets were dissolved in 1N NaOH and the protein contents were determined by using a BSA protein assay kit (Pierce).

2.8. Cultivation of the cells

EBL and T-1 cell lines maintained with periodic passages were used as test-systems. Cells were routinely grown in a 1:1 (v/v) mixture of culture medium 199 (1×), supplemented with penicillin and streptomycin (100 IU/ml) and culture medium Eagle MEM (2×), supplemented with L-glutamine and penicillin–streptomycin (100 IU/ml) with the addition of 10% bovine serum. The infection of the cells was carried out in culture medium without serum. Cells were incubated in 24- and 96-well plates at 37 °C in the atmosphere of CO_2 (5%).

2.9. Virus infection study

The infectious titer of BHV-1 was estimated as the 50% tissue culture infective dose ($TCID_{50}$) with a titration method. The initial titer of BHV-1 was $10^{6.5}$–$10^{7.5}$ $TCID_{50}$/ml. The dilutions of BHV-1 from 10 to 10^7 times in maintenance medium either without inhibitors or with the native aprotinin, BBI, or $(ole)_1$BBI, were prepared and kept for 1 h at 20 °C. Then various volumes of each dilution of virus were placed in wells of 24- (0.8 ml) or 96-well plates (0.1 ml) with cell culture. When microcapsulated aprotinin was used, each dilution of virus was placed into 24- or 96-well plates, which then were filled with culture medium, or culture medium containing microcapsules up to 0.8 and 0.1 ml, respectively. Infected cells were maintained in the culture medium for 6–8 days (EBL cells) or 15 days (T-1 cells).

The infectious titer was determined in each probe. The studies were repeated at least three times to determine represented means of replicates and mean deviations.

3. Results and discussion

3.1. Preparation and properties of oleoylated BBI

Lipidization of peptides/proteins often resulted in highly modified products with limited water solubility, decreased stability in water solution and low biological activity (Hashimoto et al., 1989; Kozlova et al., 1999). Recently, we have reported a method of polypeptides acylation by fatty acid derivatives in a mixture of organic solvents which accomplished the regulation of the number of acyl residues, and the preparation of highly active lipidized polypeptides with a high yield (Tyurina et al., 1998; Malykh et al., 2001).

In the present study, the use and optimization of this acylation procedure for BBI and *N*-hydroxysuccinimide ester of oleic acid resulted in the synthesis of BBI derivatives containing one to three oleoyl groups. The absence of the native protein in purified oleoylated BBI preparations was established with the use of electrophoresis in polyacrylamide gel (Fig. 1). Various physico-chemical properties of oleoylated BBI were studied and compared with those of native BBI. Table 1 shows that BBI conjugates containing a higher number of oleoyl residues have a lower pI value, due to a decrease in the total positive charge of the protein following acylation of the amino groups. The study of distribution of oleoylated BBI in the Triton X-114–water system demonstrated a tendency for an increase in the protein content in the organic phase along with an increase in the number of modified amino groups (Table 1). Consequently, the protein lipophilicity was enhanced.

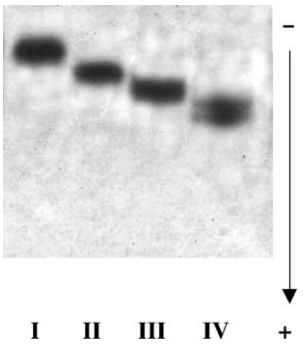

Fig. 1. Native PAGE of BBI preparations. I: BBI, II: $(ole)_1$BBI, III: $(ole)_2$BBI, IV: $(ole)_3$BBI.

Table 1
Properties of oleoylated BBI preparations

Preparation	pI	Relative *P*	Antitryptic activity (%)	Antichymotryptic activity (%)
$BBI_{(org)}$[a]	4.5	1	100	100
$(ole)_1BBI$	4.2	1.10	94	98
$(ole)_2BBI$	3.8	1.33	83	96
$(ole)_3BBI$	3.5	1.56	65	90

[a] $BBI_{(org)}$ is citraconylated BBI preparation incubated in the acylating medium without the acylating agent and then decitraconylated. Its antiproteinase activity was taken for 100%.

The study of the influence of hydrophobization on the activity of modified BBI preparations has shown that the reversible protection of trypsin-reactive site of BBI (Lys 16–Ser 17) by citraconylation ensured rather high antitrypsin activity of all the preparations (Table 1). Moreover, the BBI conjugates retained more than 90% of inhibitory activity against chymotrypsin, due to a high conformational stability of BBI in the organic medium used for the oleoylation (Malykh et al., 2001). Besides, in contrast to the antitrypsin reactive site, the antichymotrypsin reactive site of BBI (Leu 43–Ser 44) could not be directly modified with acylating agents.

Antielastase activity of oleoylated BBI preparations is of particular interest because proteinase of BHV-1 reveals elastase-like specificity cleaving two Ala–Ser bonds in the virus protein (Gao et al., 1994; Haanes et al., 1995). It is worth mentioning that oleic acid is capable of suppressing HLE activity $K_i = 9\,\mu M$ (Ashe and Zimmerman, 1977). Conjugates of BBI containing one and two oleoyl residues proved to be more potent inhibitors of HLE compared to the native BBI (Fig. 2). Whereas $(ole)_3BBI$ had a lesser affinity to HLE. The reason for some weakening of the enzyme–$(ole)_3BBI$ complex is presumably due to the steric hindrance from numerous contacts between the rather small binding "pocket" of HLE and the triacylated inhibitor.

Taking into account that the upper respiratory epithelium serves as a site of BHV-1 primary location and reproduction, we have examined cellular accumulation of oleoylated BBI using a Caco-2 cell monolayers model system. The cellular uptake of $(ole)_1BBI$ and $(ole)_2BBI$ by Caco-2 cells was 200- and 100-fold compared to the native inhibitor, respectively (Fig. 3). It was demonstrated that the $(ole)_1BBI$ had the highest affinity in the epithelial cells. Therefore, $(ole)_1BBI$ was chosen for antiviral activity evaluation.

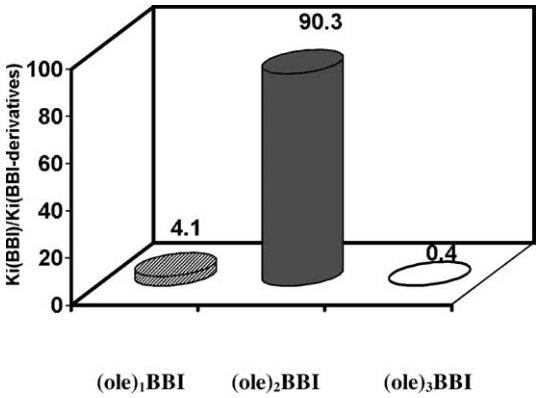

Fig. 2. Influence of the number of oleoyl residues attached to BBI on its affinity to HLE.

3.2. Microencapsulation of aprotinin

In our earlier studies we developed a rational strategy for the encapsulation of the proteinase inhibitor aprotinin. In the present work we produced, under optimal condition, stable starch/BSA microcapsules with a high yield. According to the data of scanning electron microscopy, the microcapsules had a smooth continuous surface 50–100 μm in size (Fig. 4). The protein was released from microcapsules only as a result of enzymic cleavage of their walls. Changing the manufacturing conditions can control the pharmacokinetics of protein release. Fig. 5 shows that the

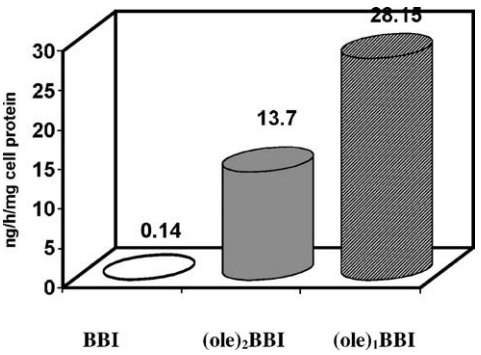

Fig. 3. Uptake of oleoylated BBI preparations by Caco-2 cell monolayers.

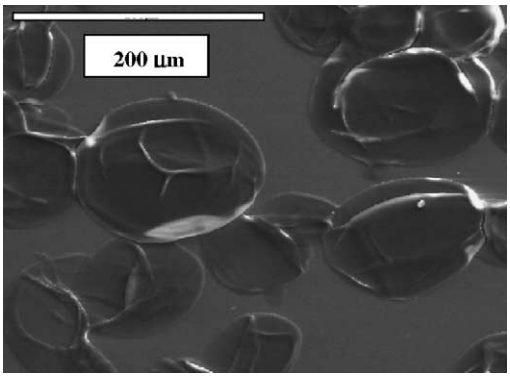

Fig. 4. Scanning electron micrograph of the lyophilized aprotinin loaded microcapsules.

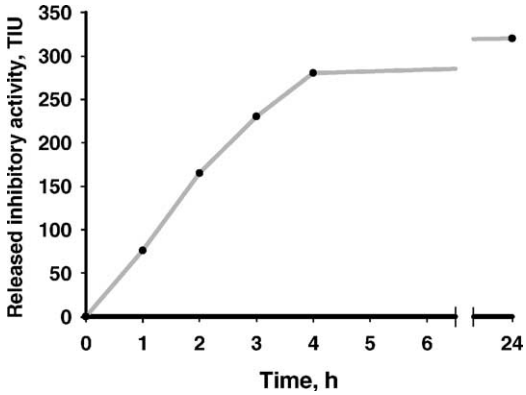

Fig. 5. Release of the antitryptic activity of aprotinin during degradation of sterilized microcapsules (10 mg) by α-amylase (0.2 mg/ml) in PBS buffer pH 7.4, 37 °C.

antitryptic activity of aprotinin was released into the medium from the sterilized microcapsules under the action of α-amylase over a 24-h period.

3.3. Antiviral effect of membranotropic and mucoadhesive formulations of BBI and aprotinin

The results of dose-dependent inhibition of BHV-1 reproduction by the various forms of proteinase inhibitors are shown in Table 2. Free aprotinin in a dose ≥800 TIU/ml inhibited reproduction of BHV-1 by 2.00–4.00 lg, whereas the native BBI had no effect even at higher doses. One of the reasons for the absence of BBI antiviral effect could be the 14 times reduction in cellular uptake compared to aprotinin (data not shown). In contrast, the lipophilic derivative $(ole)_1$BBI exhibited effective inhibition of BHV-1 reproduction. The combined effects of 200-fold increase of cellular uptake, even in the presence of serum-containing culture medium and the 90-fold increase in affinity to the elastase-like proteinase of $(ole)_1$BBI compared to the native inhibitor are most likely responsible for the observed antiviral action of $(ole)_1$BBI. Another reason for the increased efficacy of the suppression of BHV-1 reproduction by $(ole)_1$BBI could be a great improvement on stability and plasma half-life as it was found for palmitoyl-BBI (Honeycutt et al., 1996). Aprotinin encapsulated in bioadhesive, biodegradable microcapsules displayed a significantly higher inhibition of BHV-1 reproduction

Table 2
The inhibition of BHV-1 virus reproduction with formulations of proteinase inhibitors

Inhibitor	Cell culture	Formulation of inhibitor	Activity of inhibitor (TIU/ml medium)	Delay CPE (h)	Virus infectious titer decrease at $TCID_{50}$ (lg)
BPTI	EBL	Native	660	24	0
			800	24	2.0
			3300	96	4.0
		Microcapsules	500	72	2.5
			1050	144	4.0
			2000	144	4.0
	T-1	Microcapsules	2000	48	3.0
BBI	T-1	Native	2000	–	0
			3000	–	0
		$(ole)_1$BBI	2000	24	2.0
			3000	48	2.5

in diverse cell cultures than that of free inhibitor. This effect appeared to be explained by slower metabolization of the microencapsulated form of aprotinin in cell cultures than the free aprotinin. The proteinase inhibitor was gradually released in the medium during the enzymatic degradation of the microcapsules. Furthermore, aprotinin released can be bound to water soluble carrier fragments resulting from amylase action on starch/BSA mixed-walled microcapsules. There are a number of examples which demonstrate that proteins bound to water-soluble polymers are more stable to degradation processes, than the native ones (Torchilin, 1990). In particular, conjugates of the protein proteinase inhibitors with clinical dextran, CM-cellulose and hydroxyethylstarch were shown to be more stable against thermoinactivation (Larionova et al., 1999b). Moreover, aprotinin conjugates with polysaccharides were characterized by a five-fold decrease in the specific plasma clearance and a two-fold increase in the mean residence time (Larionova et al., 1999b). Importantly, our results showed that the antiviral effect of microencapsulated aprotinin was very similar in EBL and T-l cell cultures, thus supporting our hypothesis that various formulations of protein proteinase inhibitors influenced the fundamental processes of virus replication cycle.

4. Conclusions

Aprotinin possesses appreciable antiviral effect respecting to BHV-1 up to 4.0 lg in diverse cell cultures. Formulation of the inhibitor in bioadhesive, biodegradable starch/BSA microcapsules allows a decrease of the effective dose of aprotinin. Conjugation of oleic acid with BBI resulted in improvement of its membranotropic characteristics and manifestation of suppression of BHV-1 reproduction. Further investigation of the antiviral effect of various proteinase inhibitors formulations in vivo is needed to test whether they can be tools for prophylaxis and therapy for cattle.

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References

Ando, Y., Inoue, M., Utsumi, T., Morino, Y., Araki, S., 1988. Synthesis of acylated SOD derivatives which bind to the biomembrane lipid surface and dismutate extracellular superoxide radicals. FEBS Lett. 240, 216–220.

Ashe, B.M., Zimmerman, M., 1977. Specific inhibition of human granulocyte elastase by *cis*-unsaturated fatty acids and activation by the corresponding alcohols. Biochem. Biophys. Res. Commun. 75, 194–199.

Bordier, C.J., 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256, 1604–1607.

Bundgaard, H., Møss, J., 1990. Prodrugs of peptides. 6. Bioreversible derivatives of thyrotropin-releasing hormone (TRH) with increased lipophilicity and resistance to cleavage by TRH-specific serum enzyme. Pharm. Res. 7, 885–892.

Chase, T., Shaw, E., 1967. *p*-Nitrophenyl-*p*′-guanidinobenzoate HCl: a new active site titrant for trypsin. Biochem. Biophys. Res. Commun. 29, 508–514.

Chekhonin, V.P., Ryabinkin, I.A., Zhirkov, Y.A., Kashparov, I.A., Dmitriyeva, T.B., 1995. Transport of hydrophobized fragments of antibodies through the blood–brain barrier. Neuroreport 7, 129–132.

Ekrami, H.M., Kennedy, A.R., Shen, W.-C., 1995. Water-soluble fatty acid derivatives as acylating agents for reversible lipidization of polypeptides. FEBS Lett. 371, 283–286.

Fields, R., 1971. The measurement of amino groups in proteins and peptides. J. Biochem. 124, 581–590.

Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S.F., Newcomb, W.W., Brown, J.C., McCann III, P.G., Deckman, I., Colonno, R.J., 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. J. Virol. 68, 3702–3712.

Haanes, E.J., Thomsen, D.R., Martin, S., Homa, F.L., Lowery, D.E., 1995. The bovine herpesvirus 1 maturational proteinase and scaffold proteins can substitute for the homologous herpes simplex virus type 1 proteins in the formation of hybrid type B capsids. J. Virol. 69, 7375–7379.

Hashimoto, M., Takada, K., Kiso, Y., Muranishi, S., 1989. Synthesis of palmitoyl derivatives of insulin and their biological activities. Pharm. Res. 6, 171–176.

Honeycutt, L., Wang, J., Ekrami, H., Shen, W.C., 1996. Comparison of pharmacokinetic parameters of a polypeptide, the Bowman–Birk protease inhibitor (BBI), and its palmitic acid conjugate. Pharm. Res. 13, 1373–1377.

Hummel, B.C.W., 1959. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. Can. J. Biochem. Physiol. 37, 1393–1399.

Illum, L., 1999. Bioadhesive formulations for nasal peptide delivery. In: Mathiowitz, E., Chickering III, D.E., Lehr, C.-M. (Eds.), Bioadhesive Drug Delivery Systems. Marcel Dekker, New York, pp. 507–535.

Kabanov, A.V., Ovcharenko, A.V., Melik-Nubarov, N.S., Bannikov, A.I., Lisok, T.P., Klyushnenkova, E.V., Cherchenko, N.G., Alakhov, V.Yu., Levashov, A.V., Kiselev, V.I., Sveshnikov, P.G., Kiselev, O.I., Severin, E.S., 1990. Effective inhibition of viral reproduction by hydrophobised antiviral antibodies. Biomed. Sci. 1, 63–68.

Kakade, M.L., Simons, N., Liener, I.E., 1969. An evaluation of natural vs. synthetic substrate for measuring the antitrypsin activity of soybean samples. Cereal. Chem. 46, 518–526.

Kamyshny, A., Feldman, A., Boissonnade, M.M., Rosilio, V.V., Magdassi, S., 1999. Chemically modified glucose oxidase with enhanced hydrophobicity: adsorption at polystyrene, silica, and silica coated by lipid monolayers. J. Colloid Interface Sci. 218, 300–308.

Kozlova, N.O., Bruskovskaya, I.B., Melik-Nubarov, N.S., Yaroslavov, A.A., Kabanov, V.A., 1999. Catalytic properties and conformation of hydrophobized α-chymotrypsin incorporated into bilayer lipid membrane. FEBS Lett. 461, 141–144.

Kurtzhals, P., Havelund, S., Jonassen, I., Kiehr, B., Larsen, U.D., Ribel, U., Markussen, J., 1995. Albimin binding of insulins acylated with fatty acids: characterization of the ligand–protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. Biochem. J. 312, 725–731.

Larionova, N.V., Ponchel, G., Duchene, D., Larionova, N.I., 1999a. Biodegradable cross-linked starch/protein microcapsules containing proteinase inhibitor for oral protein administration. Int. J. Pharm. 189, 171–178.

Larionova, N.I., Moroz, N.A., Tyurina, O.P., 1999b. Molecular design, characterization and pharmacological activity of conjugates of protein proteinase inhibitors. S.T.P. Pharm. Sci. 9, 67–82.

Malykh, E.V., Tyurina, O.P., Larionova, N.I., 2001. Acylation of Bowman–Birk soybean proteinase inhibitor by unsaturated fatty acid derivatives. Biochemistry (Moscow) 66, 444–448.

Mc Conahey, P.C., Dixon, F.J., 1980. Radiation of proteins by the use of the chloramines-T method. Methods Enzymol. 70, 221–247.

Melik-Nubarov, N.S., Suzdaltseva, Yu.G., Priss, E.L., Slepnev, V.I., Kabanov, A.V., Zhirnov, O.P., Sveshnikov, P.G., Severin, E.S., 1993. Interaction of hydrophobised antiviral antibodies with influenza virus infected MDCK cells. Biochem. Mol. Biol. Int. 29, 939–947.

Misra, V., Blumenthal, R.M., Babiuk, L.A., 1981. Proteins specified by bovine herpesvirus 1 (infectious bovine rhinotracheitis virus). J. Virol. 40, 367–378.

Morrison, J.F., Walsh, C.T., 1988. The behavior and significance of slow-binding enzyme inhibitors. In: Meister, A. (Ed.), Adv. Enzymol. Rel. Areas Mol. Biol., vol. 61. Interscience, New York, Chichester, Brisbane, Toronto, Singapore, pp. 201–301.

Muranishi, S., Sakai, A., Yamada, K., Murakami, M., Takada, K., Kiso, Y., 1991. Novel lipophilic peptides: synthesis of lauroyl thyrotropin-releasing hormone and its biological activity. Pharm. Res. 8, 649–652.

Rathbone, M.J., Gurny, R. (Eds.), 2000. Controlled Release Veterinary Drug Delivery: Biological and Pharmaceutical Consideration. Elsevier, Amsterdam.

Reisfeld, R.A., Lewis, U.I., Williams, D.E., 1962. Disc-electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195, 281–290.

Shonbaum, J.R., Zerner, B., Bender, M., 1961. Titration of active sites of α-chymotrypsin with *N-trans*-cinnamoylimidazole. J. Biol. Chem. 236, 2930–2935.

Stein, R.L., Strimpler, A.M., Hori, H., Power, J.C., 1987. Catalysis of human leucocyte elastase: mechanistic insights into specificity requirements. Biochemistry 26, 1301–1305.

Surin, V.N., Belousova, R.V., Fomina, N.V., 1991. Diagnostics of Animal Viral Diseases. Agropromizdat, Moscow.

Tikhonova, T.V., Larionova, N.I., Gladysheva, I.P., Kazanskaya, N.F., 1993. Bowman–Birk soybean inhibitors as affinity ligands for isolation of leukocyte elastase. Inhibition of leukocytes elastase-mediated elastin hydrolysis. Biochemistry (Moscow) 58, 1227–1233.

Tirucherai, G.S., Pezron, I., Mitra, A.K., 2002. Novel approaches to nasal delivery of peptides and proteins. S.T.P. Pharm. Sci. 12, 3–12.

Torchilin, V.P., 1990. Progress in clinical biochemistry and medicine: immobilized enzymes. In: Kellen, J.A. (Ed.), Medicine. Spriner-Verlag, New York, p. 206.

Tyurina, O.P., Malykh, E.V., Balabushevich, N.G., Larionova, N.I., 1998. The modification of basic pancreatic trypsin inhibitor by fatty acid derivatieves. Russ. J. Bioorg. Chem. 24, 301–304.

Wang, J., Shen, W.-C., 2000. Gastric retention and stability of lipidized Bowman–Birk protease inhibitor in mice. Int. J. Pharm. 204, 111–116.

Wang, J., Shen, D., Shen, W.-C., 1999. Preparation, purification and characterization of a reversibly lipidized desmopressin with potentiated anti-diuretic activity. Pharm. Res. 16, 1674–1679.

Zhirnov, O.P., Ovcharenko, A.V., Bukrinskaya, A.G., 1982. Protective effect of protease inhibitors in influenza virus infected animals. Arch. Virol. 73, 263–272.

Zhirnov, O.P., Golyando, P.B., Ovcharenko, A.V., 1994. Replication of influenza B virus in chicken embryos is suppressed by exogenous aprotinin. Arch. Virol. 135, 209–216.