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Layer-by-Layer Adsorption of Biopolyelectrolytes as a Universal Approach to Fabrication of Protein-Loaded Microparticles

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Abstract—This study was aimed at examination of microparticles formed via the layer-by-layer adsorption of dextran sulfate and chitosan onto the insoluble complexes of various proteins with polyanions. Microparticles with all tested proteins were stable at pH values of 1–5. At pH > 6 the mucoadhesivity of the microparticles changed and the encapsulated proteins were released. Microparticles were able to protect the proteins from proteases. Proteinous protease inhibitors encapsulated as well (2–3%) completely prevented protein proteolysis. The pharmacological effect of microencapsulated insulin was studied in vivo using the model of chronic diabetes in rats, which were treated by oral administration.

Keywords: microencapsulation, layer-by-layer biopolyelectrolyte adsorption, proteins, proteolysis, protein delivery

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Proteins and peptides are widely used in modern medicine and the development of biotechnology significantly expands the possibilities of their application [1, 2]. Numerous studies have focused on the preparating of biocompatible and biodegradable micro-and nanoencapsulated drugs that are capable of protecting hormones and enzymes from the corrosive environment of the body and controllably releasing them in the certain conditions, mainly depending on the acidity of the medium. Particular attention is paid to the creation of oral delivery systems of protein drugs [3].

Since the 1990s, the encapsulation of labile bioactive substances on colloidal particles with different compositions and structures by layer-by-layer adsorption of oppositely charged polyelectrolytes has been widely studied [4]. The main advantage of this immobilization is the implementation of the process under mild conditions in an aqueous medium. Three main approaches to layer-by-layer microencapsulation proteins have been described [5]: the introduction of proteins in a colloidal matrix for subsequent adsorption of polyelectrolytes [6–11]; the use of proteins as polyelectrolytes in constructing multilayers [12, 13]; and the inclusion of proteins in resulting polyelectrolyte microparticles when the permeability of walls is changed [14–17].

To achieve high content of target proteins in polyelectrolyte particles the first approach, namely the use of organic or inorganic microarrays containing proteins, is the most often used. In an inorganic microarray (usually of a well-defined size) the proteins are involved during their formation or sorption onto a porous surface. Particular attention has been attracted to the highly porous microspheres of calcium carbonate, which are easily destroyed under mild conditions [9, 10]. In the absence of strict requirements on the shapes and sizes of polyelectrolyte microparticles the crystals or precipitated microaggregates of proteins have been used as microarrays for adsorption of multilayers [6–8]. However, this type of microparticles has been obtained only for a limited number of enzymes and insulin [12, 18, 19]. Insoluble complexes with polyanion proteins, which can be the basis for obtaining polyelectrolyte microparticles with desired properties, have been recognized as versatile microarrays [5]. The average microparticle size is 3–10 microns and the pH-sensitive properties fulfil the requirements for oral delivery [12, 20–24].

As known from [25], an oral delivery method is associated with overcoming the greatest number of barriers to blood flow, which significantly reduces the bioavailability of proteins. The pH in an empty human stomach is 1.1–2.0 . The food remains in the stomach for 4.0–5.0 h at pH 0.5–5.0. In the small intestine the pH varies from 5.0 to 8.0 for 4 to 6 h. Furthermore, proteins are susceptible to proteolysis. They are digested in the stomach by pepsin, in the intestines by trypsin, chymotrypsin, and elastase of pancreatic juice. In the small intestine proteins must penetrate the glycocalyx, a sulfated mucopolysaccharide layer

Table 1. The properties of microparticles with protein produced by adsorption of chitosan and dextran sulphate ($s = 3$)

Encapsulated protein	pI	M_w, kDa	Protein encapsulation efficiency, %	Content, %			Size, μm
				protein	dextran sulfate	chitosan	
Ovomucoid	3.8	28	15 ± 1	n/d	n/d	n/d	4 ± 3
BBI	4.2	8.0	62 ± 5	55 ± 4	27 ± 3	17 ± 2	5 ± 1
Ovalbumin	4.7	45	54 ± 6	58 ± 5	30 ± 6	7 ± 2	3 ± 1
Insulin aspart	5.1	5.8	64 ± 5	56 ± 2	27 ± 1	16 ± 3	8 ± 5
Human insulin	5.5	5.8	68 ± 4	57 ± 2	25 ± 2	16 ± 5	9 ± 4
Pig insulin	5.5	5.8	72 ± 5	52 ± 4	34 ± 6	9 ± 2	9 ± 5
Chymotrypsin	8.8	25	59 ± 3	49 ± 3	32 ± 8	14 ± 2	9 ± 3
Trypsin	10.5	24	63 ± 5	63 ± 1	24 ± 5	8 ± 2	7 ± 3
Aprotinin	10.5	6.5	43 ± 2	39 ± 8	41 ± 5	13 ± 2	8 ± 3
Lactoferrin	11.5	73	85 ± 5	75 ± 3	15 ± 3	8 ± 3	7 ± 3

and a layer of mucus, which consists of glycoproteins (mucins), enzymes, electrolytes, and water, and then must overcome the epithelial barrier. Selection of biopolymers of certain compositions and structures for microencapsulation by layer-by-layer adsorption of polyelectrolytes allows particles to add properties that increase the bioavailability after oral administration of proteins [5].

The aim of our work was to assess the universality of the approach of preparing the microparticles by layer-by-layer adsorption of biopolyelectrolytes on an insoluble complex of polyanions with the protein, as well as to study the protective effects of microparticles on the possible processes that occur when they are administered to a living organism in vitro and in vivo. Under in vivo conditions we studied the effects of microencapsulated insulin when administered orally to rats with experimental diabetes.

To provide the bioavailability of insulin we chose the biocompatible and biodegradable macromolecular polycation chitosan and polyanion dextran sulfate. The mucoadhesive properties of chitosan facilitate fixation of microparticles of the intestinal mucus layer, increasing the contact time with the intestinal lining [26, 27]. Chitosan increases the intercellular permeability of the intestinal epithelium by interacting with proteins of tight cell–cell junctions [26, 28]. Dextran sulfate binds calcium ions and thereby further promotes the opening of these junctions [29].

Protection of microencapsulated proteins from proteolysis in the small intestine should be provided by pH-sensitive microparticles [23]. The following well-known protein inhibitors of trypsin, chymotrypsin, and elastase were included in the microparticles along with the target proteins: soybean Bowman–Birk inhibitor (BBI) and aprotinin, with inhibition constants (K_i) for trypsin, chymotrypsin, and elastase, respectively, equal to 9×10^{-9}, 6.4×10^{-9}, and 2.0×10^{-9} and 6×10^{-14}, 1.5×10^{-9}, and 3.5×10^{-6} M [13].

To evaluate the universality of the approach ten therapeutically important proteins that are different with respect to their physico-chemical and biological properties were chosen as encapsulated proteins: ovomucoid from duck eggs, hen egg albumin (ovalbumin), porcine insulin, recombinant human insulin and its analogue that is not susceptible to hexamerization (aspart with a ProB28 substitution with Asp), human lactoferrin from milk, and the above-mentioned protease inhibitors and proteolytic enzymes (trypsin and chymotrypsin) (Table 1).

MATERIALS AND METHODS

Materials. In this work, we used sodium dextran sulfate ($M_w = 500$ kDa), aprotinin from bovine lung and BBI containing 46 and 55%, respectively, of the active inhibitor, porcine insulin, porcine pepsin (4500 U/mg), ovalbumin, mucins (type I-S and type III), N-benzoyl-L-tyrosine ethyl ester (BTEE), N-benzoyl-L-tyrosine-paronitroanilide (BTPNA) (Sigma, USA); chitosan, $M_w \approx 400$ kDa, with a degree of deacetylation of 85%, α-chymotrypsin (61 U/mg) from (Fluka, Switzerland); trypsin (40 U/mg), N-benzoyl-L-arginine ethyl ester (BAEE) (Merck, Germany); and Sephadex G-50, G-75 superfine (LKB-Pharmacia, Sweden). Recombinant human insulin and aspart as zinc salts were prepared in the M.M. Shemyakin and Yu.A. Ovchinnikov Institute of bioorganic chemistry of the Russian Academy of Sciences. The lactoferrin from human milk was obtained in the P.A. Gertsen Moscow Scientific and Research Oncological Institute. The ovomucoid from duck eggs (Ovomin), which contains 51% of the active inhibitor, was produced in the Belmedpreparaty RUE (Belarus). Other reagents were of chemically pure and extra pure grade.

Preparation of polyelectrolyte microparticles. To obtain the microaggregates of an insoluble polyanion–protein complex at pH 3.0, equal volumes of protein solution (20 mg/mL) and dextran sulfate (5 mg/mL)

were mixed, stirred for 20 minutes, and then centrifuged for 3 minutes at 200 g. In a 0.15 M NaCl solution (pH 3.0) chitosan ($s = 2$), dextran sulfate ($s = 3$), and again chitosan ($s = 4$) were sequentially adsorbed (2.5 mg/mL) on microaggregates until the desired number of stages of sorption (s), as described in [20]. The microparticles were then suspended in a solution of 0.15 M NaCl (pH 3.0) and stored at 4°C and washed three times with 1 mM HCl and freeze dried. If necessary, the microparticle suspension was sonicated using a Sonopuls HD 2070 homogenizer (Bandelin, Germany).

Microparticle characterization. Microparticle morphology was studied using an Olympus FV300 confocal microscope (Japan) and a Leo Supra 50VP scanning electron microscope (Carl Zeiss, Germany) with an accelerating voltage of 3–5 kV with an increase to 5000–150000. The average size of the microparticles was determined by a Carl Zeiss Opton III optical microscopy (Germany) by measuring 100 particles. In determining the composition of microparticles the protein was analyzed by the Lowry method [30] and dextran sulfate by the Dubois method [31]; chitosan was determined by reacting the amino groups with *o*-phthalic aldehyde *N*-acetyl-*l*-cysteine [32]. Trypsin and chymotrypsin activities were measured using BAEE [33] and BTEE [34], respectively; the activity of aprotinin was determined by trypsin inhibition [33] and BBI was found by chymotrypsin inhibition using BTPNA [35]. Preparations were first suspended in 0.1 M NaOH; for determining their activity at 1 min after suspension, the samples were diluted (1 : 10) by 0.5 M Tris buffer solution (pH 7.8).

Protein release from microparticles. The suspension of microparticles was mixed with universal buffer (0.02 M H_3PO_4; 0.02 M CH_3COOH; and 0.02 M H_3BO_3 + 0.1 M NaOH; pH 2–8) to a final protein concentration of 0.20–0.25 mg/mL and incubated for 1 h with stirring (100 rpm) at room temperature. Samples were then centrifuged for 1 min at 10000 g; the protein concentration was measured in the supernatants. Protein release was evaluated in relation to its concentration values in the supernatant and in the suspension of microparticles. In the study of the kinetics a microparticle suspension containing 1 mg of protein was mixed with solution of HCl at pH 1.1 to a total volume of 4 mL and incubated for 8 h with stirring (100 rpm) at room temperature. After 2 and 4 h from the start of experiment the suspension was centrifuged for 5 min at 160 g, the supernatant was collected, and the same volume of 0.05 M Na-phosphate buffer at a pH of 6.0 and 7.4, respectively, was added to the residue. The suspension aliquots were taken and centrifuged for 5 min at 1000 g every hour after beginning the process. Concentrations of protein and protease inhibitors were determined in the supernatants.

Chromatography (on a column of 1 × 13 cm) on Sephadex G-50 or G-75, respectively, for the low-molecular weight and high-molecular weight proteins was used to study the release forms of the protein from microparticles. When preparing the samples, a suspension of microparticles and 0.05 M Na-phosphate buffer (pH 7.4) were mixed to a protein concentration of 2 mg/mL, incubated for 2 hours, and centrifuged; 0.5 mL of the supernatant was applied to the column. The retention times in the column for dextran sulfate, native microparticles, and microparticles freed from the protein were compared.

Proteolytic degradation of the protein. The suspensions of microparticles (0.5 mg/mL of protein) were incubated at 37°C for 2 h in a 0.08 M HCl solution containing 0.034 M NaCl and 0.1 mg/mL pepsin [36], or 1 h in 0.05 M tris-buffered saline (pH 7.1) containing 700 U/mL trypsin and 4 U/mL chymotrypsin [37]. Proteolysis was stopped by addition of trifluoroacetic acid to a concentration of 0.1%; the samples were centrifuged and the supernatants were frozen and then analyzed by HPLC on a C18 column (4 × 250 mm) with detection at 210 nm. The eluent was a mixture of 0.1% TFA in water and 0.1% TFA in acetonitrile; the ratio of components was changed in a gradient during 5 min from 80/20 to 50/50%, respectively and the flow rate of the eluant was 1 mL/min. Degradation of insulin was assessed by the ratio of the areas of peaks corresponding to insulin before and after exposure to proteases.

Mucin adsorption on microparticles: 1.6 mL of a 0.25 mg/mL mucin solution was added to 2 mg of the dried microparticles. The suspension was incubated for 1 h at 37°C (100 rpm). The supernatant was separated by centrifugation (5 min, 1500 g) and the mucin content was analyzed using Schiff's reagent [38]. Mucin adsorption was calculated from the difference between the added and the remaining amount of mucin in the supernatant.

Ca^{2+} binding by microparticles. One mL of 0.01 M $CaCl_2$ solution was added to 0.2 mL of a suspension of microparticles (12.7 mg/mL). The mixture was incubated for 30 min (100 rpm) and centrifuged (5 min, 1200 g). The content of Ca^{2+} was determined by titration with EDTA 2 in the supernatant solution using eryochrom black T [39].

In vivo evaluation of the biological activity of microparticles. The study was performed on male Wistar rats that weighed 250–300 g, which were kept at a temperature of 22 ± 2°C on a standard diet and with free access to water and food. Diabetes was induced by intraperitoneal administration of streptozotocin at 50 mg/kg body weight (a solution of 26.6 mg/mL in 0.1 M citrate buffer, pH 4.5). After 14 days, two groups were formed from animals with a fasting glucose level of at least 14 mM: 0.1 mL of microencapsulated insulin suspension in a solution of 0.15 M NaCl (pH 3.0) was administered orally (every 6 h) to the test group (seven animals). A solution of 0.15 M NaCl (pH 3.0) was also administered to the control group (six animals). The single dose of microencapsulated insulin for 4 days was 100 U/kg. The glucose level was evalu-

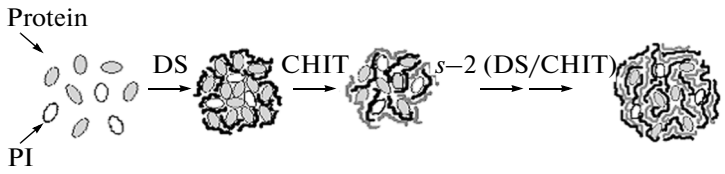

Fig. 1. Scheme for obtaining microparticles by sequential adsorption of polyelectrolytes on microaggregates of insoluble complex protein and polyanion (DS, dextran sulfate; CHIT, chitosan; PI, protease inhibitor; *s*, number of stages of the sorption of polyelectrolytes).

ated against the background of feeding ad libitum. Point 0 h corresponded to the glucose level in the morning before drug administration; point 1 h corresponded to the glucose level at 1 h after injection and point 6h to the glucose level after 6 h after the first daily injection of the drug. The preparation was then administered to the animals the second time.

Sampling of blood was performed in the rats from the tail vein. For the determination of glucose in animals, 20 mL of blood was collected and measurements were carried out using a OneTouchR II glucometer and OneTouchR stripes (USA). For determination of the content of human insulin 100 mL of blood was collected in heparinized tubes, centrifuged (20 min, 400 g), the supernatant was separated and stored frozen, and after thawing the enzyme immunoassay was performed (Insulin Elisa Monobind Ins, 413-8103 (USA)).

Statistical analysis of the results. The results are presented as the mean ± standard deviation. Statistical processing of the results was carried out using the Statistica 6.0 software package.

RESULTS AND DISCUSSION

Characterization of microparticles. Microencapsulation was performed at pH 3.0 (0.15 M NaCl) in two steps (Fig. 1). At the first step under these conditions all the proteins had a positive charge (Table 1) and formed insoluble complexes with dextran sulfate with an average of two to three sulfate groups at each link. At the second step, chitosan and dextran sulphate were adsorbed stepwise onto the microcomplexes to achieve the desired number of steps of sorption (*s*). When dextran sulphate was used in the last step of microparticle production, these microparticles had a negative surface charge, but when chitosan was used, they had a positive surface charge. As shown in our previous papers, $s = 3–4$ is enough to produce stable particles [20]. The effectiveness of inclusion of all proteins in the microparticles exceeded 50% (Table 1) except for ovomucoid with a pI of 3.8, which is slightly positively charged at pH 3.0, and low-molecular weight aprotinin, which is more prone to the formation of soluble complexes with dextran sulfate [21]. The protein content in the microparticles was the highest and the encapsulated proteases and protease inhibitors retained high levels of activity (trypsin 44 ± 4%, chymotrypsin 66 ± 4%, BBI 98 ± 2%, and aprotinin 99 ± 1%).

Along with microparticles that contained only one protein, microparticles that contained the target protein and protease inhibitor were obtained. To accomplish this, during the formation of an insoluble complex with dextran sulfate a mixture of protein and aprotinin or IBB was added. The ratio of protein and protease inhibitor, which was chosen in our previous works pursuant to the actual content of proteases in the biological fluids of the gastrointestinal tract, was 1 : 20. [9] The composition of microparticles with $s = 4$ with recombinant insulin and protease inhibitor is shown in Table 2.

The prepared polyelectrolyte microparticles with $s = 3$ (Table 1) and $s = 4$ (Fig. 2) were formations with irregular shapes and sizes of 3–9 microns. Using sonication, the microparticle size was reduced to 3.1 microns. Microparticles with all proteins did not aggregate in the suspension during storage within 2 years of observation. After freeze drying and subsequent resuspension the positively charged microparticles with $s = 4$ preserved an average size greater than the negatively charged microparticles with $s = 3$.

Table 2. The composition of microparticles that were obtained by adsorption of chitosan and dextran sulphate ($s = 4$) to an insoluble complex of insulin and a protease inhibitor (20 : 1)

Protein type		Content, %			
insulin	protease inhibitor	insulin	protease inhibitor	dextran sulfate	chitosan
Human	BBI	52 ± 6	2.7 ± 0.3	30 ± 5	15 ± 5
Human	Aprotinin	54 ± 5	2.2 ± 0.2	24 ± 4	20 ± 5
Aspart	BBI	53 ± 5	2.5 ± 0.3	29 ± 4	14 ± 6

We investigated the mucoadhesive properties of the microparticles on the binding surface of the main part of the small intestinal mucosa mucin glycoprotein, which contains 0.5–1.5% (type III) or 9–17% (type I–S) of sialic acids. As seen in Table 3, regardless of the content of sialic acid, mucin was better connected to the contact with the surface of the positively charged microparticles $s = 4$, recharging from +(30–34) to –(40–43) mV.

The pH-sensitive and protective properties of the microparticles. The results of the evaluation of the pH stability of the microparticles with $s = 3$ are shown in Fig . 3. Release of model proteins from microparticles was observed at pH > 6. The most active transfer to the solution occurred with acidic proteins (ovalbumin, BBI, and insulin). Basic proteins (chymotrypsin, trypsin, aprotinin, and lactoferrin) were released at higher pH values. At the same time, proteases and protease inhibitors completely preserved their activity. According to gel permeation chromatography the releasing from microparticles acidic proteins remained in their native form, while basic proteins were found in the form of a soluble complex with dextran sulphate (data not shown).

The differences in the values of the release rates of acidic and basic proteins were taken into account with simultaneous inclusion of the protease inhibitors along with the target protein into microparticles and proteins were selected with similar isoelectric points. As can be seen from Fig. 4, when modeling the change in pH during passage of the human gastrointestinal tract the curves of the release from microparticles with $s = 4$ of insulin and BBI with acidic pI values differ from the curves of the release of aprotinin and lactoferrin, which have basic pI values. Upon complete release of proteins the insoluble residues of microparticles consisting of dextran sulfate and chitosan had a ζ-potential equal to 39 ± 2 mV. Thus, their mucoadhesive properties must be reduced to a level characteristic of microparticles with $s = 3$ (Table 3), which facilitates the removal of residues from the intestine.

The microparticles should not only release the target proteins at given pH values, but also protect them from proteolysis. When simulating the action of pepsin for 2 hours in a 0.08 M solution of HCl, destruction of the microparticles in all experiments with model proteins was not observed. During subsequent destruction of the microparticles at pH of 7.4 and study of the released proteins by chromatographic methods, a complete lack of proteolysis of human insulin and proteolysis insulin aspart was shown.

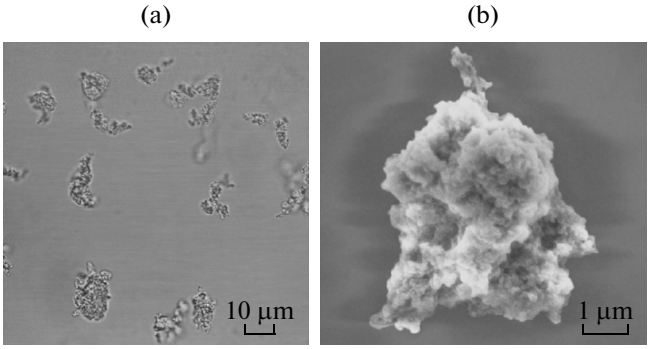

Fig. 2. Confocal laser scanning (a) and scanning electron (b) micrograph of microparticles with human insulin produced by adsorption of chitosan and dextran sulphate ($s = 4$).

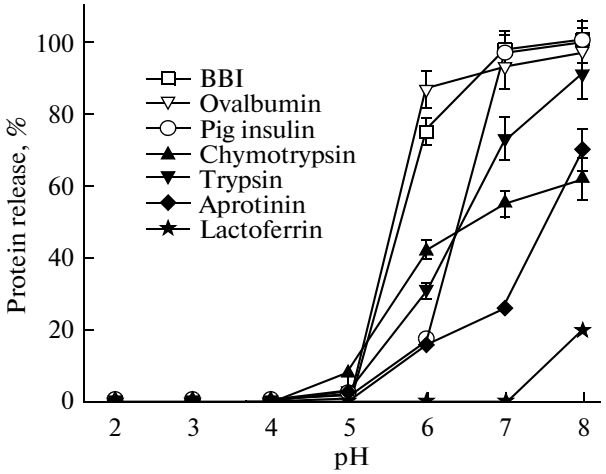

Fig. 3. Effect of pH on the release of proteins from the microparticles prepared by adsorption of chitosan and dextran sulphate ($s = 3$). Incubation time is 1 h.

The results of studies of human proteolysis of insulin, which exists primarily in the form of a hexamer and is more susceptible to proteolysis than monomeric insulin aspart, in a medium containing trypsin and chymotrypsin in high concentrations and modeling human pancreatic juice (pH 7.1) [37] are shown in Fig. 5. Both recombinant insulins were completely cleaved in solution. The proteolysis values of microencapsulated human insulin and insulin aspart were 60 and 78%, respectively.

The protective properties of the microparticles were due to the gradual release of the target protein

Table 3. Mucin binding with human insulin by microparticles that were obtained by adsorption of chitosan and dextran sulfate

Number of stages of adsorption of polyelectrolytes (s)	ζ-potential of microparticles, mV	Adsorption of mucin, μg/mg of particles	
		type III	type I-S
3	–40 ± 1	17 ± 10	24 ± 8
4	32 ± 2	86 ± 15	50 ± 1

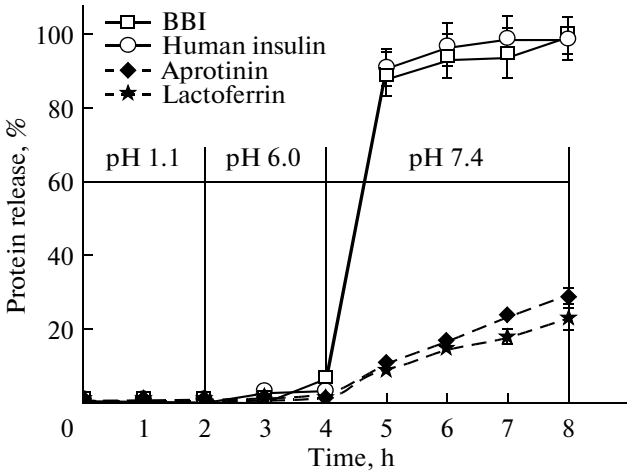

Fig. 4. Kinetics of the pH-dependent release of the target protein and protease inhibitor of microparticles obtained by adsorption of chitosan and dextran sulfate ($s = 4$): open symbols, microparticles with human insulin and BBI; closed symbols, microparticles with lactoferrin and aprotinin.

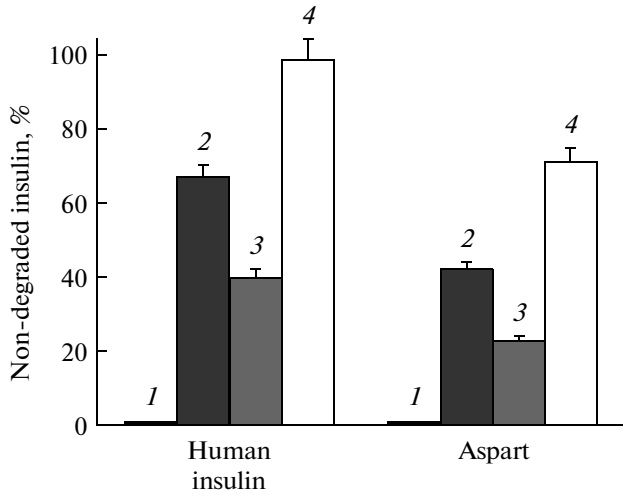

Fig. 5. The influence of BBI on the proteolysis of recombinant human insulin and aspart (0.5 mg/mL) under conditions that simulate the pancreatic juice (700 U/mL trypsin, 4 U/mL chymotrypsin, 1 h, pH 7.1 (*1*, insulin; *2*, BBI and insulin (20 : 1); *3*, microencapsulated insulin; *4*, microencapsulated BBI and insulin (20 : 1)).

from the microparticles and calcium ion binding by the microparticles (10 ± 0.5 4 ± 0.4 μg/mL, respectively, for $s = 3$ and $s = 4$), which affects the activity of calcium-dependent trypsin [40]. For a 2–3% solution of BBI, the proteolysis of human insulin was 35%, while that of insulin aspart was 58% (Fig. 5). A similar amount of BBI in the microparticles completely prevented cleavage of encapsulated human insulin and reduced proteolysis of insulin aspart by 29% compared to reactions in solution.

The pharmacological action of microparticles with insulin in vivo. The action of microparticles ($s = 4$) containing human insulin was studied in a model of streptozotocin-induced diabetes in rats. The results of determining the concentration of human glucose and insulin in the blood of animals with constant ingestion are shown in Fig. 6[1]. Data were obtained by averaging the results of 3 consecutive days of the experiment. A significant decrease in the blood glucose level ($p < 0.05$ by the Wilcoxon test) was observed 1 hour after injection of encapsulated insulin at a dose of 100 U/kg to the animals of the test group (Fig. 6a). At 1 h after administration of the encapsulated drug the absolute values of glucose in the blood of animals were significantly lower than in the control animals that had not received insulin. The administration of microencapsulated insulin to animals in the test group significantly increased the content of insulin in the blood plasma (relative to the initial level $p < 0.05$ by the Wilcoxon test) after 1 h and then after 6 h. These values are significantly higher (Fig. 6b) than the respective indices in the test group despite the cross reaction of antibodies with rat insulin ($p < 0.05$ by the Mann–Whitney test).

When administered at the same dose of microparticles containing insulin and BBI further reduction of the concentration of glucose in the blood of animals after 1 h by 7 ± 2% occurred, as compared with the preparation that did not contain protease inhibitor.

Thus, microparticles were obtained with ten proteins with different biological and physico-chemical properties by layer-by-layer adsorption of the oppositely charged dextran sulphate and chitosan on the insoluble dextran sulfate–protein complexes. Except for ovomucoid from duck eggs with a pI of 3.8, which is close to the pI of the formation of an insoluble complex with a polyanion, the microparticles were obtained with all proteins with a high inclusion efficiency of target protein (40–70% protein, 15–40% dextran sulfate, and 10–20% chitosan). Comparison of microparticles with three ($s = 3$) and four ($s = 4$) stages of adsorption of polyelectrolytes revealed that microparticles with $s = 4$ and positively charged chitosan on a surface were better suspended after freeze drying. Furthermore, they have more pronounced mucoadhesive properties, which is important to ensure adhesion of the microparticles to mucous membranes [27].

In the formation of an insoluble microcomplex with polyanion in the microparticles along with a target protein the protein protease inhibitors may be administered in the desired ratio. The selection of a protease inhibitor was performed by considering the proximity of its isoelectric point with the same value for the target protein, which is favorable for their simultaneous gradual release.

Polyelectrolyte microparticles were stable in an acidic environment and protected encapsulated proteins from the action of pepsin. The release of the pro-

[1] $p < 0.05$ by the Wilcoxon test (the reliability of differences from the 0 point), while $p < 0.05$ by the Mann-Whitney test (significance of differences between groups).

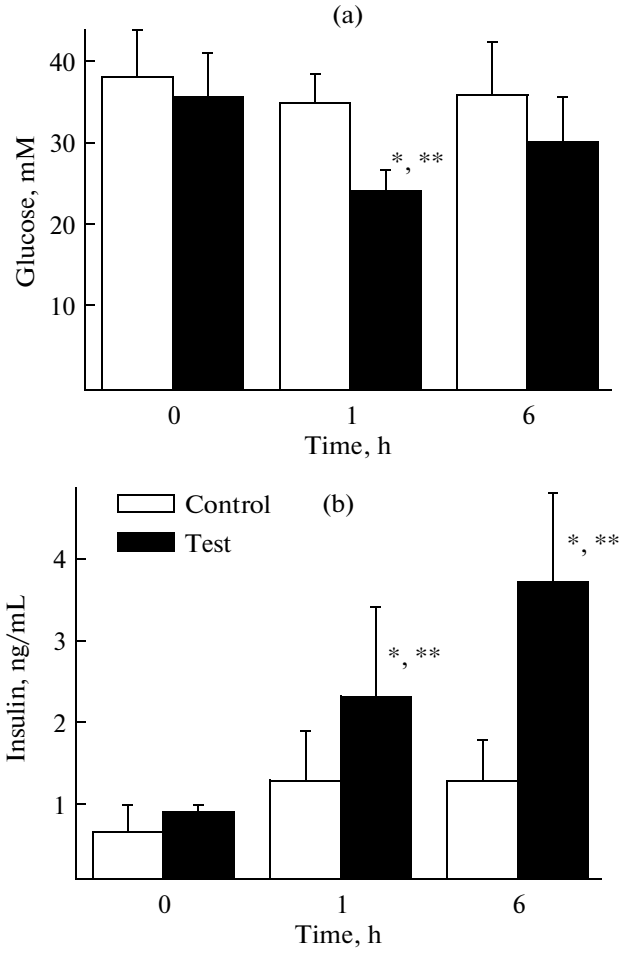

Fig. 6. Concentration of glucose (a) and insulin (b) in the blood of diabetic rats after oral administration of the microparticles with human insulin (100 U/kg) to the animals of the test group and the buffer solution to the control group animals. Measurements were performed under the conditions of feeding ad libitum.

teins was observed from the microparticles at pH > 6. Acidic protein release took place in the native form faster than in the basic form of soluble complex with the dextran sulfate.

Microparticles protected freed target proteins from the actions of the major proteases. Additional injection of the proteinous protease inhibitor into the microparticles (less than 2–3%) with a pI close to the pI of the target protein allows almost complete preventing of its proteolysis in biological fluids that contain trypsin and chymotrypsin in high concentrations.

In a model of diabetes in rats with administered orally at a dose of 100 U/kg, presence of microencapsulated insulin human insulin in plasma after 1 and 6 h and a decrease of the level of glucose in the blood of animals after 1 h were demonstrated. In the conditions of the experiment with constant access to food and drink, these results confirm the protective effect of microparticles against proteolysis of a target protein by enzymes of the gastrointestinal tract.

Thus, in this work the universality of the approach of microencapsulation of proteins by layer-by-layer adsorption of biopolyelectrolytes on an insoluble protein–polyanion complex was demonstrated. It was shown that such polyelectrolyte microparticles have an ability to protect the target protein from external proteases, including those in biological fluids. The protective effects of protease inhibitors that were incorporated in microparticles simultaneously with a target protein were investigated.

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