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DRUG SYNTHESIS METHODS AND MANUFACTURING TECHNOLOGY

USE OF PROTEASE INHIBITORS IN COMPOSITE POLYELECTROLYTE MICROPARTICLES IN ORDER TO INCREASE THE BIOAVAILABILITY OF PERORALLY ADMINISTERED ENCAPSULATED PROTEINS

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Protein protease inhibitors (aprotinin, soybean Bowman–Birk inhibitor, and Kunitz soybean trypsin inhibitor) possessing different specificity with respect to trypsin, chymotrypsin, and elastase were encapsulated together with a cargo protein in polyelectrolyte microparticles using layer-by-layer (LbL) deposition techniques. The most efficient inclusion of the inhibitors occurred at the formation stage of the insoluble protein complex with the polyanion. Simultaneous immobilization of the inhibitor and protein did not influence the physicochemical properties of the microparticles, specifically their pH-sensitive behavior under conditions modeling the passage through various parts of the human gastrointestinal tract after peroral administration. The most effective protection against the action of proteolytic enzymes of pancreatic juice and the small intestine was achieved for simultaneous release of cargo protein and inhibitor from the microparticles. Soybean Bowman–Birk inhibitor, which is most similar to insulin with respect to physicochemical properties, in addition to the soybean extract enriched with protease inhibitors were the most suitable agents for protection of human insulin or its rapidly acting analogs (lispro and aspart). These findings suggested that simultaneous microencapsulation of both protein and protein protease inhibitor was a promising way to increase the protein bioavailability upon peroral administration of polyelectrolyte microparticles.

Keywords: microparticles, layer-by-layer deposition of polyelectrolytes, protein protease inhibitors, insulin, peroral administration of proteins.

Patients are most comfortable with peroral administration of drugs. Therefore, the development of peroral drug delivery systems for proteins and peptides continues to be critical. The small intestine is the most preferred site for protein adsorption because of the large surface area (200 – 500 m^2) [1]. The low bioavailability of proteins and peptides by peroral administration is due to hydrolysis in the stomach, low permeability of intestinal epithelium for the rather large molecules, and proteolysis in the gastrointestinal tract (GIT).

Proteins can be protected from aggressive stomach fluids by inclusion in stimulus-sensitive particles, in particular, in pH-sensitive polymeric matrices [2]. Muco-adhesive polymers that ensured proteins were released when the carrier was in close proximity to the site in the intestine where it was adsorbed were used to overcome diffusion complications [3]. It is well known that proteins in the free state can penetrate the intestinal lumen into the blood circulation due to *trans*- and *para*-cellular transport or within nanoparticles owing to capture by M-cells of Peyer's patches [4].

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Small-intestine fluids contain a large amount of proteolytic enzymes. Thus, the ratio of trypsin to chymotrypsin and elastase, which are released by the pancreas into the intestine lumen after food intake, was estimated as about 20:1:2 [5]. A total of about 45 g of these enzymes were secreted during one day. Several strategies for suppressing protein proteolysis were proposed. The simplest was delivery of proteins as complexes with polymers capable of reducing protease activity [6-9]. The capability of several polymers or matrices based on them to bind Ca^{2+}, reducing the activity of Ca^{2+}-dependent serine proteases, was used [6, 10, 11]. An original approach with the protein-loaded matrix covered by albumin was reported [12]. The albumin became the target of the proteases whereas the cargo protein remained protected. However, these approaches enable protein hydrolysis to be minimized but not avoided.

One of the most effective ways to battle proteolysis is to include protease inhibitors (PI) in the matrices containing protein drugs. Different effectiveness of PI activity was found experimentally for low-molecular-weight molecules [13, 14] and proteins such as aprotinin [15, 16], ovomucoid [17, 18], soybean Bowman–Birk inhibitor [19], and their mixtures [20]. High PI doses were usually used. In several instances this could disrupt digestion. In the overwhelming majority of instances, this problem was overcome by covalent binding of the PI to the polymeric matrix [13, 20, 21]. This decreased their reactivity by hindering diffusion and did not enable proteins released from the matrix to be protected. These drawbacks could be avoided by non-covalent inclusion of the PI within the protein-carrier matrix such as micro- and nanoparticles.

The goal of the present work was to study the incorporation of PI proteins into biological polyelectrolyte microparticles containing cargo proteins. Attention was focused on solving the problem of increasing the bioavailability of encapsulated proteins by decreasing their enzymatic hydrolysis in the GIT. This was achieved by using the pH-sensitive properties of the microparticles or by introducing different protein PI. The microparticles were prepared by layer-by-layer deposition of oppositely charged polyelectrolytes (LbL technique in English) on a matrix consisting of an insoluble complex of the protein with the polyanion [10, 22 – 25]. Undisputable advantages of the selected encapsulation method were the high incorporation effectiveness (up to 70%) and protein content (up to 60%) in the microparticles in addition to the pH-dependent release of proteins from them [22, 25]. The well-known biocompatible and biodegradable polysaccharides dextransulfate (DS) and chitosan (Chit) were used as the polyelectrolytes. This imparted good mucoadhesive properties to the microparticles [10]. According to *in vivo* results from s.c. injection to healthy rabbits, insulin in solution and insulin encapsulated in microparticles of the selected polyelectrolytes had the same biological activities [26]. Microparticles formed from DS and Chit reduced significantly proteolysis of insulin under conditions modeling peroral delivery [23, 26].

We used recombinant human insulin and its rapidly acting analogs insulin aspart, which replaces Pro^{B28} by Asp, and insulin lispro, in which the two amino-acid residues in the 28 and 29 positions were reversed (Pro^{B28} by Lys and Lys^{B29} by Pro) (Table 1). In contrast with human insulin, both analogs do not form hexamers and; therefore, are more susceptible to cleavage by proteases [27]. Furthermore, we used well-known protein PI that were capable of inactivating the major proteases of the human small intestine. These included aprotinin (Apr), Bowman–Birk inhibitor (BBI), and ovomucoid (Ovo) (Table 1). The inhibitors differed in pI values, molecular weights, and effectiveness of small-intestine proteinase inhibition. Apr has one active center and inhibits most effectively trypsin. BBI possesses two active centers, one of which binds trypsin; the other, chymotrypsin. Of the three active centers of Ovo, two bind trypsin and one, chymotrypsin. BBI and Ovo are also effective inhibitors of elastase. In addition to the expensive preparations of these inhibitors, soy protein extract (BBI/KI extract) enriched in BBI and Kunitz inhibitor (KI), which has two trypsin binding centers capable of binding (to a lesser extent) chymotrypsin, was investigated (Table 1).

EXPERIMENTAL PART

We used recombinant human insulin (Ins), aspart, and lispro as the zinc salts (Shemyakin and Ovchinnikov IBC, RAS, Russia); the preparations Ingiprol, which contained 52% active bovine lung aprotinin (Apr), and Ovomin, which contained 51% active ovomucoid from duck eggs (Ovm, PO Belmedpreparaty, Belarus); BBI from soy, which contained 51% active inhibitor, DS (MW 500 kDa), porcine pepsin (4500 U/mg), *N*-benzoyl-L-arginine ethyl ester (BAEE), *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA), and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma, USA); chitosan (Chit, MW 400 kDa) with 85% deacetylation, α-chymotrypsin (61 U/mg, 48% active centers), and trypsin (40 U/mg, 61% active centers) (Fluka, Germany).

Soy flour was extracted at pH 3.0 and precipitated by Me_2CO at pH 5.3 in order to produce protein extract enriched in BBI/KI [35]. The precipitate was dissolved in H_2O and ultrafiltered on a UPM-10 membrane (NPO Vladipor). The concentrate was lyophilized. The protein extract contained 90% protein including 32% trypsin inhibitors, 9% chymotrypsin inhibitors, and 6% carbohydrates.

Preparation of microparticles with insulin

Microparticles were prepared in HCl solution (1 mM) containing NaCl (0.15 M) [10, 26]. Various volumes of solutions of insulin (20 mg/mL) and DS (5 mg/mL) were mixed, stirred vigorously for 20 min, and centrifuged for 2 min at 200 g in order to produce microaggregates of the insoluble complex (Ins–DS). The precipitate of microaggregates were resuspended twice in HCl solution (1 mM) containing NaCl

(0.15 M) and centrifuged. Then, LbL deposition on the aggregates of the insoluble complex (Ins–DS) was carried out by successive treatment with solutions of Chit (2.5 mg/mL), DS (2.5 mg/mL), and Chit (2.5 mg/mL). The microparticles were incubated in each step for 10 min with polyelectrolyte solution. The precipitate was separated by centrifugation (2 min, 200 g) and rinsed twice. The suspension of microparticles was stored at 5°C or rinsed twice with HCl solution (1 mM) and lyophilized.

Incorporation of PI into microparticles with insulin. PI were incorporated during formation of microaggregates of the insoluble complex with DS by mixing solutions of insulin and PI before reaching the required Ins:PI mass ratio and final protein content 20 mg/mL. Then, microaggregates and microparticles were produced as described above.

Sequential treatment by PI (2.5 mg/mL), DS, and Chit solutions was carried out in order to incorporate PI during polyelectrolyte deposition during LbL deposition on microaggregates of the insoluble Ind:DS complex.

Characteristics of microparticles

Protein concentration was determined by the Lowry method [36]; DS concentration, by the Dubois method [37]; Chit concentration, by reaction of the amine with *o*-phthalaldehyde and *N*-acetyl-L-cysteine [38]; of the inhibitors, by titration of the active centers using trypsin or chymotrypsin for BAEE [39] and BTPNA [40], respectively.

Lyophilized preparations were suspended in NaOH solution (0.1 M) and the protein and polysaccharide concentrations were determined in order to determine the composition of the microparticles. The biological activity of the inhibitors were determined by suspending the suspension or lyophilized microparticle preparations in NaOH solution (0.1 M) to concentration 1 mg/mL, diluting 20× with Tris-buffer (0.05 M, pH 7.8), centrifuging, and determining the PI concentration in the supernatants. Insulin content in the particles with immobilized Apr or BBI was determined by subtracting the PI content from the total protein content. The percent contents of the components in the microparticles were defined as the ratio of the measured amounts of the separate components to the mass of the lyophilized preparation. The incorporation effectiveness of insulin and PI into the microparticles was defined as the ratio of the amount of protein in the microparticles to the amount of protein used to produce them.

The average microparticle diameter was determined by optical microscopy (Opton III, Carl Zeiss, Germany) using measurements of 100 particles. The zeta-potential of the microparticles was measured in KCl solution (0.01 M, pH 3.0) on a laser electrophoresis instrument (Malvern Zetasizer II, Malvern Instruments, Great Britain).

Release of proteins from microparticles

The effect of pH on PI and insulin release was found by treating a suspension of microparticles with universal buffer (0.02 M H_3PO_4, 0.02 M CH_3COOH, 0.02 M H_3BO_3 + 0.1 M NaOH, pH 2-8) to final protein concentration 0.20 – 0.25 mg/mL, and stirring for 1 h at 100 rpm and room temperature. Samples were centrifuged for 5 min at 10,000 g. The protein and PI concentrations in the supernatants were analyzed. The release was estimated from the concentration ratio of microparticles in the supernatant and in the suspension.

The protein release kinetics from a suspension of microparticles was studied by stirring (100 rpm) and incubating successively in HCl solution (pH 1.1, protein concentration 0.20 – 0.25 mg/mL) for 2 h; in phosphate buffer (0.05 M, pH 6.0) for 2 h; and in phosphate buffer (0.05 M, pH 7.4) for 4 h. The suspension was centrifuged for 2 min at 200 g during a buffer change. The supernatant was separated. The precipitate was treated with a volume of new buffer analogous to that of the supernatant. Aliquots of the suspension were taken during the whole incubation process. These were centrifuged for 5 min at 10,000 g. The protein and PI concentrations in the supernatants were determined.

The form of the protein release from the polyelectrolyte microparticles was determined by chromatography in phosphate buffer (0.05 M, pH 7.4) over a column of Sephadex G-50sf (1 × 13 cm) that was calibrated beforehand using Ins, Apr, BBI, and DS. Samples were prepared by mixing a suspension of microparticles and phosphate buffer (0.05 M, pH 7.4) before reaching protein concentration 2 mg/mL, incubating for 2 h, centrifuging, and placing supernatant (0.5 mL) onto the column. The optical density at wavelength 280 nm and the DS and PI concentrations in the fractions were analyzed. The ratio of the analyzed parameter to its total value in all fractions at the column outlet was found for each fraction taking into account the volume.

Proteolytic degradation of insulin

Proteins were dissolved and microparticles were suspended to a final protein concentration 0.5 mg/mL and incubated at 37°C (100 rpm) in one of the artificial media modeling separate sections of the GIT, e.g., in stomach juice (HCl, 0.08 M; NaCl, 2 mg/mL, pepsin, 0.1 mg/mL) for 2 h [41]; in pancreatic juice (Tris-buffer, 0.05 M, pH 7.1; 700 BAEE-units of trypsin/mL; 4 BTEE-units of chymotrypsin/mL) for 1 h [20]; or in lower small-intestine lumen section juice (Tris-buffer, 0.05 M, pH 7.8; 140 BAEE-units of trypsin/mL) for 4 h [42]. The activities in the trypsin (9000 BAEE units/mg) and chymotrypsin (39 BTEE units/mg) preparations were measured as described before [39, 43]. Proteolysis of pepsin was established by adding NaOH solution (5 M) to pH 8.0; of trypsin and chymotrypsin, by adding trifluoroacetic acid (TFA) to concentration 0.1%. Then, the

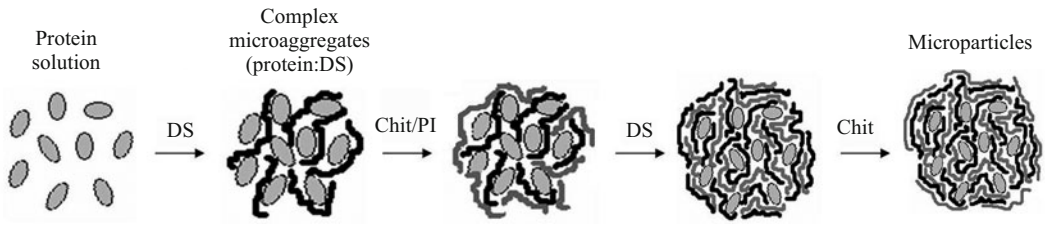

Fig. 1. General diagram for preparation of protein-containing polyelectrolyte microparticles.

mixtures were centrifuged for 5 min at 10,000 g. The supernatant was collected and analyzed using HPLC over a C-18 column (4 × 250 mm) with detection at 210 nm. The eluent was a mixture of TFA in H_2O (0.1%) and TFA in CH_3CN (0.1%). The ratio of the components was changed in a gradient over 5 min from 80/20% to 50/50%. Insulin degradation was estimated from the ratio of peak areas corresponding to intact insulin before and after the action of the proteases.

RESULTS AND DISCUSSION

Incorporation of proteins into polyelectrolyte microparticles

Insulin and its analogs were microencapsulated at pH 3.0 [10, 26]. The process was carried out in two steps. First, nanostructured insoluble polyelectrolyte complexes (IPC) of DS with human Ins as the Zn-salt hexamer or its analogs as the Zn-salt monomers were produced. Then, LbL deposition of Chit, DS, and Chit again was carried out (Fig. 1). PI were incorporated into the microparticles in two ways. These were adding them during formation of IPC of insulin and DS or in the second deposition step using them as the polycation instead of Chit.

Mixing an Ovm solution with a DS solution did not form an insoluble complex. In all probability, the relatively low pI value of this glycoprotein (3.8), which did not differ greatly from the pH of the medium for preparing the microparticles, was responsible for an exceedingly insignificant positive charge on the protein and, as a result, a weak interaction with the polyanion. Inhibitors Apr, BBI, and BBI/KI from soy extract, which had higher pI values (Table 1), formed IPC at mass ratios Ins:PI from 40:1 to 10:1. The effectiveness of PI incorporation was 97 – 99%, which was close to the effectiveness of insulin incorporation [10]. It was important that the activities of all inhibitors after IPC destruction were retained completely.

Microparticles prepared from IPC and containing PI, like microparticles with insulin, had a positive ζ-potential (~+30 mV) and average size 6 ± 3 μm. The insulin and BBI incorporation effectivenesses were practically the same and reached about 60%; those of Apr and BBI/KI extract that were determined from trypsin and chymotrypsin inhibition were slightly less (Table 2). The incorporation effectiveness of all PI into microparticles increased with increasing Ins:PI ratio (Table 2). Like in our previous studies [26], the principal protein losses during microparticle production occurred because of its displacement during sorption of Chit onto the IPC. Apr was more susceptible than BBI or insulin to such displacement [23]. The component composition of the microparticles varied little and was 52 – 58% Ins, 24 – 30% DS, and 14 – 20% Chit. The Apr and BBI contents varied from 1.5 to 4%, increasing with increasing amount of PI used to form the IPC. The total contents of BBI and KI from soy extract in the microparticles for Ins:PI = 10:1 reached 14.3% and 4.2%, respectively, as measured by trypsin and chymotrypsin inhibition.

The other method for PI incorporation into the microparticles, i.e., adding inhibitor instead of Chit in the second deposition step, was studied using Apr as an example (Table 3). The effectiveness of such incorporation was much lower (10 ± 2%). Addition of significant amounts of Apr was

TABLE 1. Physicochemical Properties of Proteins Used for Microencapsulation

Protein	M_W, kDa	pI	Ki, M		
			Trypsin	α-Chymotrypsin	Elastase
Aprotinin	6.5	10.5	6×10^{-14} [28]	1.5×10^{-9} [29]	3.5×10^{-6} [29]
Ovomucoid	28	3.8	6.1×10^{-9} [30]	2.2×10^{-9} [30]	2.4×10^{-9} [30]
Bowman–Birk inhibitor	8	4.2	9×10^{-9} [31]	6.4×10^{-9}[31]	2×10^{-9} [31]
Kunitz inhibitor	20.1	4.5	3.7×10^{-9} [32]	1.0×10^{-6}; 0.3×10^{-6} [33]*	0.2×10^{-6} [34]
Human insulin (as Zn-salt hexamer)	5.8 (35)	5.35	–	–	–
Aspart insulin	5.8	5.1	–	–	–
Lispro insulin	5.8	5.35	–	–	–

* Data for two enzyme binding sites

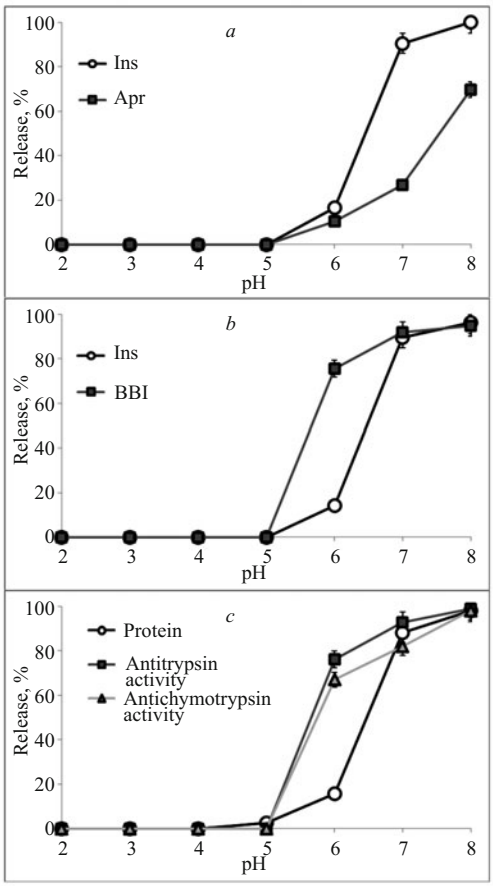

Fig. 2. pH-induced protein release from insulin-containing polyelectrolyte microparticles with immobilized Apr (*a*), BBI (*b*), and BBI/KI (*c*).

required in order to create analogous Ins:PI ratios in the microparticles (Table 2). One of the reasons for the observed reduced incorporation effectiveness may have been the difference of the protein distribution throughout the microparticle. Whereas the amount of immobilized protein upon incorporation during IPC formation was on average proportional to the particle volume (cube of the diameter), the amount of protein adsorbed on the surface of the already formed particles would be proportional to the square of their diameter, i.e., significantly less.

Release of proteins from polyelectrolyte microparticles

Simultaneous release of Ins and PI from the microparticles was monitored by the medium pH change (Fig. 2). PI molecules, which had lower pI values than Ins, were released faster than the hormone at pH > 5. Apr was released from the microparticles slower than insulin. This was due to its high pI value.

Release of proteins upon a medium pH change [41] during passage of the microparticles through the human GIT was monitored using the same factors (Fig. 3). The polyelectrolyte microparticles remained stable for 2 h at pH 1.1, i.e., under conditions modeling the stomach. Proteins were not released from the microparticles at pH 6.0, corresponding to the acidity of the upper intestinal sections in which the concentration of proteolytic enzymes produced by the pancreas is highest. At pH 7.4, corresponding to the acidity of the middle and lower small-intestine sections, BBI and BBI/KI were released analogously to Ins whereas Apr was released more slowly. According to gel filtration (Fig. 4), BBI, like Ins, was released as the free protein whereas Apr was released as a polyelectrolyte complex with DS.

Protective action of microparticles from protein proteolysis

The ability of the microparticles to protect Ins from proteolysis was studied by the three ways recommended in the literature. First, the action of pepsin in stomach juice was studied for 1 – 2 h; second, the combined action of trypsin and chymotrypsin, for 1 h at the highest concentrations corresponding to pancreatic juice at pH 7.1; and finally, the action of trypsin, for 4 h under conditions corresponding to small-intestine middle sections at pH 7.8. Keeping in mind the effectiveness of PI action, Ins:PI mass ratios of 10:1 and 20:1 for Apr and 40:1 for BBI were chosen. Solutions of Ins and PI prepared at the same ratios were used as the controls.

TABLE 2. Characteristics of Insulin-containing Microparticles with PI Incorporated During IPC Formation

PI	Ins:PI (mass)		Incorporation effectiveness, %		Content, mass%			
	During preparation	In particles	Ins	PI	Ins	PI	DS	Chit
–	–	–	65 ± 2	–	57 ± 4	–	26 ± 3	17 ± 2
Apr	20:1	25.4:1	64 ± 4	50 ± 5*	54 ± 5	2.2 ± 0.2*	24 ± 4	20 ± 5
Apr	10:1	13.4:1	62 ± 5	46 ± 5*	52 ± 4	3.9 ± 0.4*	27 ± 6	17 ± 6
BBI	40:1	37.9:1	62 ± 4	65 ± 7**	56 ± 5	1.5 ± 0.2**	25 ± 5	18 ± 6
BBI	20:1	19.6:1	60 ± 6	61 ± 6**	52 ± 6	2.7 ± 0.3**	30 ± 5	15 ± 5
Extract BBI/KI	20:1	22.4:1	54 ± 5	50 ± 5*/47 ± 5**	56 ± 5#	7.8 ± 0.8*/2.2 ± 0.3**	30 ± 4	14 ± 6
Extract BBI/KI	10:1	12.0:1	56 ± 6	46 ± 5*/45 ± 5**	58 ± 4#	14.3 ± 1.5*/4.2 ± 0.4**	28 ± 4	14 ± 6

* Determined from trypsin inhibition; ** determined from chymotrypsin inhibition; # total protein content.

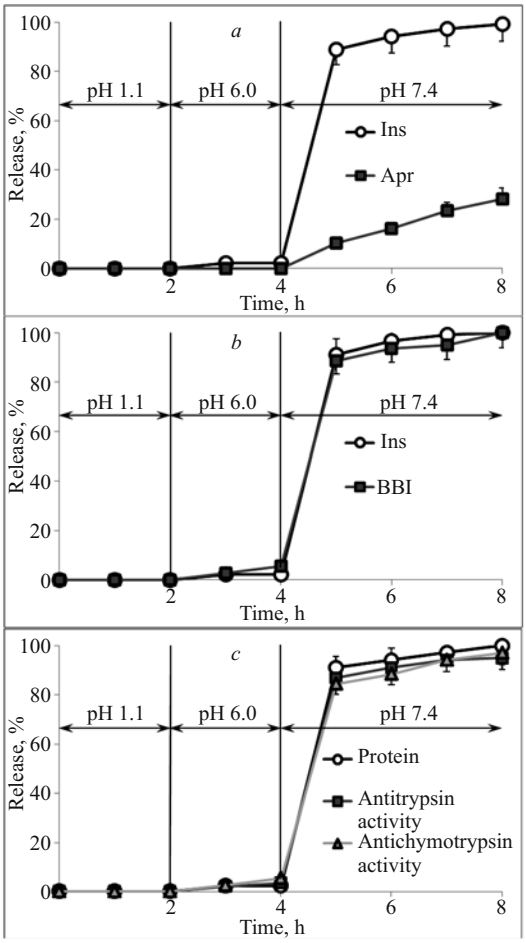

Fig. 3. Protein release kinetics from insulin-containing polyelectrolyte microparticles with immobilized Apr (*a*), BBI (*b*), and BBI/KI (*c*) under conditions modeling passage through the human GIT.

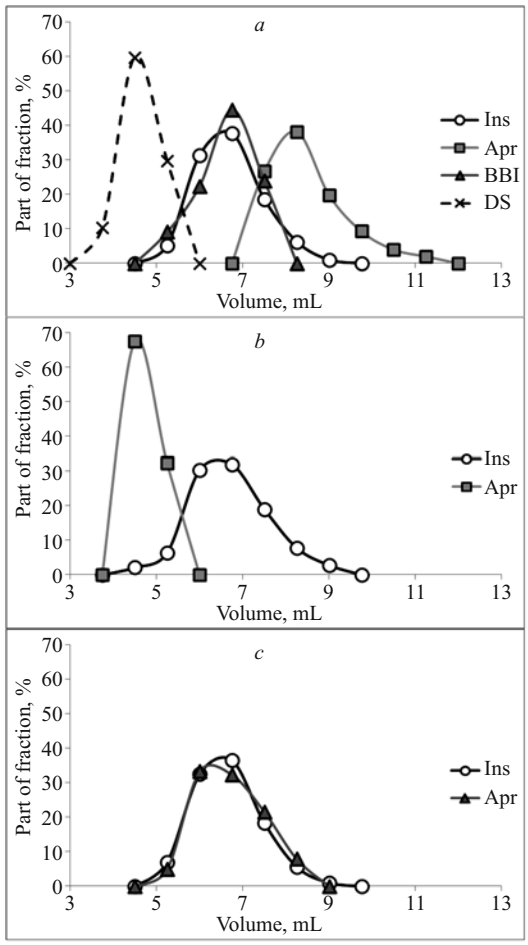

Fig. 4. Gel filtration of DS and native proteins (*a*) and proteins released from insulin-containing microparticles with immobilized Apr (*b*) and BBI (*c*). Sephadex G-50, pH 7.4.

Insulin in solution was 99% cleaved already in 1 h under conditions corresponding to stomach juice whereas microencapsulated insulin persisted in the microparticles for at least 2 h. After the incubated particles were destroyed, it turned out that only 2% of the hormone was degraded.

Insulin in solution was fully degraded under pancreatic conditions (pH 7.1) in the presence of trypsin and chymotrypsin with two and five sites, respectively, for hormone degradation (Fig. 5*a*) [44]. Apr at a ratio of 10:1 prevented more effectively than BBI at a ratio of 40:1 degradation of Ins in solution. Use of microparticles at analogous Ins:PI ratios had a more pronounced protective effect for BBI. Apparently, the difference in the release kinetics of Ins and PI from the microparticles played the decisive role at a high protease concentration and with a relatively short incubation time. A significant fraction of the Apr molecules did not manage to be released during 1 h and; therefore, did not prevent insulin proteolysis. Conversely, BBI and the delivered protein were displaced at similar rates. This determined the effectiveness of this inhibitor.

The presence of PI weakened by 40 – 45% the action of proteases on insulin in solution under conditions modeling the prolonged action of trypsin in middle and lower small-intestine sections (Fig. 5*b*). Incorporation of the same amounts of PI into the microparticles could practically completely protect the hormone from proteolysis.

The effectiveness of BBI action, which possesses two active centers and has a protective effect against proteases in smaller amounts than Apr, was studied in microparticles with aspart and lispro insulins. Because both fast-acting analogs were released from the microparticles as monomers [10], they were more susceptible to hydrolysis by proteases than human insulin that was released as a hexamer. The microencapsulated insulin analogs were practically completely destroyed under conditions corresponding to pancreatic juice (Table 4). The presence of BBI incorporated into the microparticles at a 20:1 protein:BBI ratio weakened the action of proteases on aspart and lispro by 48 and 43%, respectively. It should be noted that increasing the amount of BBI from 1:40 to 1:20 in microparticles with human insulin

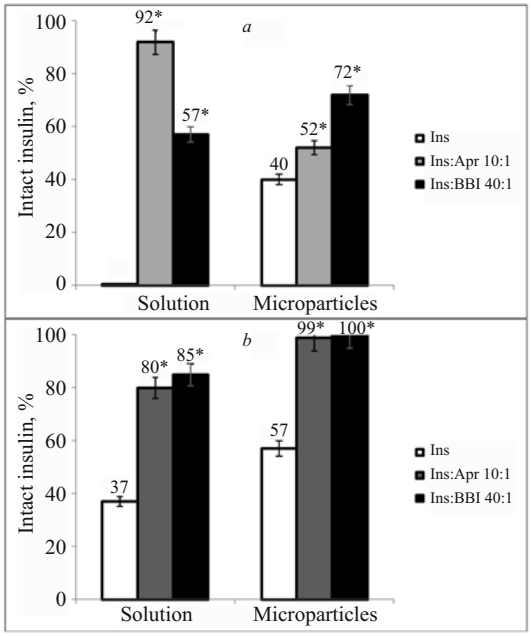

Fig. 5. Degradation of insulin (0.5 mg/mL) in solution and in polyelectrolyte microparticles by proteases: trypsin 700 BAEE U/mL, chymotrypsin 4 BTEE U/mL, pH 7.1, 1 h (*a*); trypsin 140 BAEE U/mL, pH 7.8, 4 h (*b*). * Statistically significant differences relative to the control without inhibitor ($p < 0.01$, according to Mann–Whitney criterion).

increased the amount of non-degraded hormone from 72 to 99% (Fig. 5*a*, Table 4).

Thus, PI were most effectively incorporated into polyelectrolyte microparticles with insulin at pH 3 during formation of IPC with the proteins. PI that had a significant positive charge at pH 3 (BBI, Apr, and soy protein extract enriched in BBI and KI) turned out to be most suitable for preparing microparticles of the studied protein PI.

Incorporation of PI together with cargo protein did not change the pH-sensitive properties of the microparticles under conditions modeling the human GIT. Simultaneous release from the microparticles of cargo protein and PI was most effective for protection from the action of proteases of pancreatic juice. This was observed for BBI or soy protein extract enriched in this inhibitor. The study of insulin and its fast-acting analogs aspart and lispro found that the amount of

TABLE 3. Characteristics of Insulin-containing Microparticles with Apr Incorporated in the Second Polyelectrolyte Deposition Stage

Ins:PI (mass)		Content, mass%		
During preparation	In particles	Ins	Apr	DS
20:1	130:1	65 ± 3	0.5 ± 0.1	21 ± 4
10:1	80:1	64 ± 4	0.8 ± 0.1	16 ± 2
5:1	30:1	67 ± 4	2.2 ± 0.3	15 ± 2

TABLE 4. Degradation by Trypsin and Chymotrypsin of Recombinant Insulins Encapsulated in Polyelectrolyte Microparticles Under Conditions Modeling Pancreatic Juice

Insulin	Intact insulin, %	
	Particles with insulin	Particles with insulin and BBI (20:1)
Human	40 ± 2	99 ± 5*
Aspart	23 ± 1	71 ± 4*
Lispro	0	43 ± 2*

* Statistically significant differences relative to the control without inhibitor ($p < 0.01$, according to Mann–Whitney criterion)

PI had to be increased during encapsulation of proteins that were more susceptible to proteolysis. However, this was less than 5% of the incorporated protein if BBI was used.

Because proteolysis is one of the three principal reasons for the loss of biological activity of proteins upon peroral delivery [2], incorporation of PI into polyelectrolyte microparticles should also facilitate increased bioavailability of the delivered proteins.

General principles for the behavior of polyelectrolyte microparticles of DS and Chit containing a therapeutically important protein for peroral administration were formulated. The microparticles protect the encapsulated protein from the action of acidic stomach juice that contains pepsin. The outer Chit layer ensures close contact of the microparticles with the epithelium mucosa. Simultaneous and gradual release of PI and delivered protein from the microparticles occurs during passage through the small intestine (with increasing pH). Local release of PI should protect the protein from the action of proteases of pancreatic juice but not disrupt digestion. Release of protein in the active state near the mucous surface of the small intestine facilitates its transport into the blood circulation.

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