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Inclusion of Proteins into Polyelectrolyte Microparticles by Alternative Adsorption of Polyelectrolytes on Protein Aggregates

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Abstract—A new method of protein immobilization into polyelectrolyte microparticles by alternative adsorption of the oppositely charged polyelectrolytes on the aggregates obtained by salting out of protein is proposed. The model protein α-chymotrypsin (ChT) was included in the polyelectrolyte microparticles obtained by various number of polyelectrolyte adsorption steps (from 1 to 11). The main parameters of ChT inclusion into microparticles were calculated. Scanning electron and optical microscopy were used for characterization of morphology and determination of particle size which was from 1 to 10 μm in most cases. It was shown that the size and shape of protein-containing particles and protein aggregates used as a matrix were similar. Change in ChT enzymatic activity during entrapment into polyelectrolyte particles and activity of released protein were studied. The effect of pH on release of incorporated proteins was investigated; it was shown that change in pH and the number of polyelectrolyte adsorption steps allows protein release to be manipulated.

Key words: polyelectrolyte microparticles, proteins, polyelectrolytes, poly(styrene sulfonate) sodium salt, poly(allylamine hydrochloride), α-chymotrypsin

Immobilization of proteins in polyelectrolyte complexes is of great scientific and practical importance. Encapsulation of some materials of non-protein nature, such as inorganic and colloidal particles, by alternative adsorption of oppositely charged synthetic and natural polyelectrolytes on the insoluble matrix was recently described [1-3]. This process is rather simple and is performed in aqueous solutions under mild conditions.

There are conditions which are necessary when working with biologically active substances. Protein precipitation by salting out is often used in preliminary enzyme purification. The physicochemical basis of this process is rather complex, and usually specific conditions are needed for each protein [4]. However, protein and enzyme precipitation by salting out from aqueous solutions allows the formation of aggregates with preserved biological activity and high yield.

In the present work we studied the possibility in principle of protein incorporation into polyelectrolyte microparticles by alternative adsorption of oppositely charged polyelectrolytes; the aggregates obtained by salting out of proteins were used as a matrix [5-7]. To study the process of adsorption and the properties of the obtained microparticles, α-chymotrypsin (ChT) was used as a model protein. Of the variety of polyelectrolytes we chose sodium poly(styrenesulfonate) (PSS) and poly(allylamine) hydrochloride (PAA) which are widely used for formation of polyelectrolyte envelops by alternative adsorption of polyelectrolytes.

MATERIALS AND METHODS

Reagents. The following reagents were used in this study: bovine α-chymotrypsin with activity 68 U/mg from Fluka (Germany); PSS and PAA with molecular masses 70 and 60 kD, respectively, from Aldrich (USA); Tris from Serva (USA); ethyl ester of N-benzoyl-L-tyrosine (BTEE) from Sigma (USA); Sephadex G-75 from Pharmacia (Sweden).

Preparation of microparticles. To 0.5 ml of ChT solution (35 mg/ml) in 0.01 M HCl an equal volume of saturated NaCl solution (5.4 M) and dry NaCl were added to the final NaCl concentration 5 M. After 20 min the

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formed aggregates were centrifuged (200*g*, 5 min) and the supernatant was removed.

To obtain the microparticles, PSS solution (10 mg/ml) in 5 mM HCl containing 2.7 M NaCl was added to the precipitate. Suspension was incubated with intensive stirring on a shaker for 20 min and centrifuged; the supernatant was removed and the precipitate was resuspended in 0.01 M HCl containing 2.7 M NaCl. The procedure was repeated twice to remove unbound polyelectrolyte. Then the same operation was performed for PAA, but after the first step of PSS adsorption application and washing of polyelectrolytes were performed under the same conditions but in the presence of 0.5 M NaCl. Polyelectrolytes were applied alternatively to achieve the needed number of adsorption steps.

The microparticles thus obtained were placed in 1 mM HCl or suspended in water and lyophilized. The samples were stored at 4°C.

Characterization of microparticles. The morphology of microparticles was studied by optical and scanning electron microscopy using an Option III microscope from Carl-Zeiss, Jena (Germany) and a Hitachi S-520 electron microscope (Japan, 20 kV), respectively. Particle size distribution was presented as dependence of particle percentage on their size obtained by optical microscopy.

Protein concentration. The protein concentration in solutions and suspensions of microparticles was determined according to Lowry [8]. In the lyophilized preparations the protein concentration corresponded to the ratio of protein mass to dry preparation mass.

Assay of esterase activity of α-chymotrypsin. ChT activity was assayed using BTEE as the standard [9]. For this, 0.8 ml of 0.05 M Tris-buffer, pH 8.0, and 0.1 ml of ChT solution (0.02 mg/ml) in 1 mM HCl were mixed in the cuvette and then 0.1 ml of BTEE solution (3 mg/ml) in methanol was added. Increase in optical density was monitored spectrophotometrically at $\lambda = 256$ nm against the control sample with 0.1 ml of 1 mM HCl instead of the protein solution.

Effect of pH on protein release from microparticles. An aliquot of suspension of microparticles was mixed 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 3-11) to the final protein concentration 0.20-0.25 mg/ml and incubated for 1 h with stirring (150 rpm). Then the samples were centrifuged, and the protein concentration was determined in the supernatant. Protein release from microparticles was calculated as the percent ratio of protein concentration in the supernatant to that in suspension of microparticles.

Kinetic study of protein release from microparticles. To suspension of microparticles 0.05 M Tris-buffer, pH 8.0, was added to the final protein concentration 0.20-0.25 mg/ml. This mixture was stirred at 100 rpm and aliquots were taken after 10 and 40 min and 2, 4, and 24 h. The samples were centrifuged and the protein concentration was determined in the supernatant.

When studying stepwise release of protein on pH change, microparticles were incubated in 0.05 M Tris-buffer, pH 8.0, for 1 h, then they were centrifuged for 5 min at 500*g*, and the supernatant was removed. Precipitated microparticles were resuspended and incubated in 1 mM HCl for 1 h. After centrifugation the protein concentration was determined in the supernatants.

RESULTS AND DISCUSSION

ChT immobilization into the microparticles. Since preparation of protein aggregates is the first step in ChT immobilization into the polyelectrolyte microparticles, the conditions for ChT precipitation by salting out were preliminarily optimized by the yield and retention of esterase activity. As a result, the following salting mixture was chosen: 17.5 mg/ml ChT, 5 M NaCl, and 5 mM HCl. The ChT aggregates as particles of irregular form with dimensions mainly from 1 to 10 μm retaining 86% enzymatic activity were thus obtained with 63% yield (Fig. 1). While studying the properties of aggregates it was shown that they dissolve in solutions with low ionic strength and neutral or alkaline pH. That is why the initial step in polyelectrolyte adsorption is performed in acidic medium and at high NaCl concentration to prevent premature dissolution of aggregates. Under these conditions, aggregates of ChT with p*I* 8.5 are positively charged; that is why the first adsorbed polyelectrolyte was the polyanion, PSS. Microparticles formed after application of PSS are stabilized so that they do not dissolve in acidic solutions with low ionic strength, thus subsequent steps in polyelectrolyte adsorption were performed in 1 mM HCl contain-

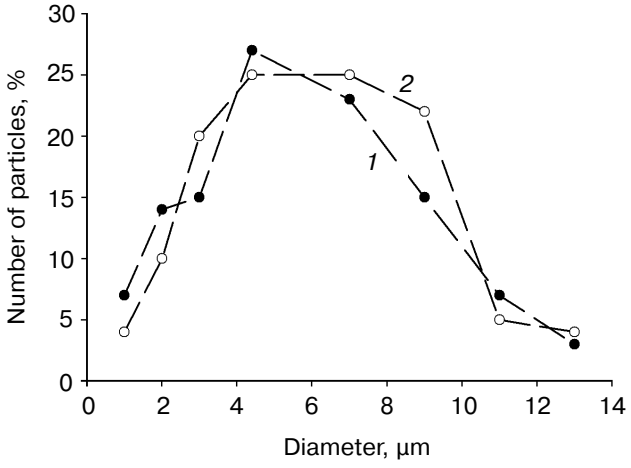

Fig. 1. Size distribution of ChT aggregates (*1*) and polyelectrolyte 11-layer microparticles (*2*) based on them.

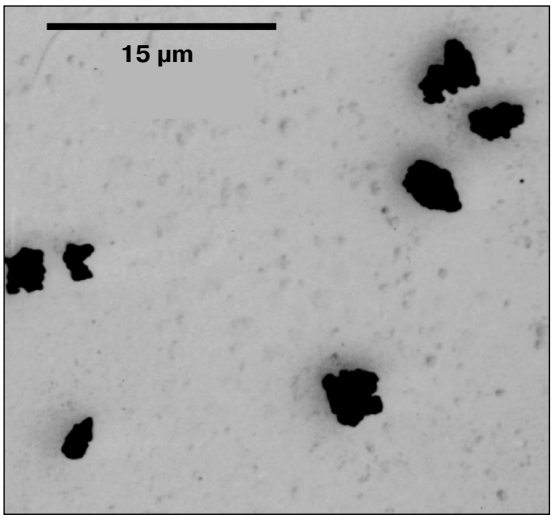

Fig. 2. Microphotography of 11-layer polyelectrolyte microparticles obtained using a scanning electron microscope.

ing 0.5 M NaCl. Next for simplicity sake let us call the number of polyelectrolyte adsorption cycles the number of polyelectrolyte layers.

Microscopic studies of 11-layer polyelectrolyte microparticles showed that they are closed particles of irregular form (Fig. 2). Comparing the size distribution curves of ChT aggregates and microparticles (Fig. 1), one can see that the size of polyelectrolyte particles is defined by the size of salted protein aggregates. Thus, the size of microparticles is defined by the initial size of insoluble matrix in protein aggregate form.

Characteristics of polyelectrolyte microparticles containing ChT. The main properties of microparticles with various number of adsorbed polyelectrolyte layers are presented in the table.

Efficiency of protein incorporation defined as the ratio of the protein content in suspension of microparticles to the initial protein used for salting out decreased moderately with increase in the number of polyelectrolyte layers. ChT aggregates obtained by salting out can be incorporated into the polyelectrolyte particles with high yield (70-80%). High protein content in lyophilized preparations decreasing with increase in the number of polyelectrolyte layers indicate that polyelectrolyte particles are formed.

Microparticles were demonstrated to be stable under the optimal conditions of ChT storage in 1 mM HCl, pH 3.0, during not less than 7 days. Being placed in solution, the lyophilized preparations remain stable during a year.

Release of α-chymotrypsin from microparticles. Studies of pH effect on stability of polyelectrolyte microparticles demonstrated that at pH less than 4 the protein is almost not released (Fig. 3) and at pH > 5 an intensive protein release is observed; this fact indicates that the microparticles are pH-sensitive.

For trilayer microparticles, protein release increases less significantly with pH increase. This is probably caused by additional protein binding with two additional polyelectrolyte layers.

It is interesting that microparticles are able to respond to alternating and multiple pH change from 3, when protein is not released from microparticles, to 8, when ChT release is observed (Fig. 4). On 1 h incubation of 11-layer microparticles at pH 8.0 almost 20% of the protein is released into solution. On subsequent replacement of microparticles into solution with pH 3.0, only about 2% of the protein is released during 1 h, that is, protein release is terminated. Stepwise protein release is observed if these operations are repeated. Thus, protein release from microparticles is reversible and pH-dependent.

The enzymatic activity of protein released from trilayer microparticles is 21% of that of the native ChT. It should be noted that this activity is almost the same as that of ChT aggregates covered with one PSS layer. Thus, application of two additional polyelectrolyte monolayers does not influence activity of protein stabilized by the first PSS layer.

As demonstrated by gel-penetrating chromatography, the protein is released from trilayer microparticles not in native form but as a complex with polyelectrolytes (Fig. 5a).

Properties of polyelectrolyte microparticles containing ChT

Number of applied layers	Efficiency of immobilized protein, % of		Protein content in lyophilized preparation, %	Protein release by 1 mM HCl during 7 days, %
	initial protein	aggregated protein		
1	53	84	80	12
3	44	70	65	2
5	45	71	67	1
11	42	67	51	0

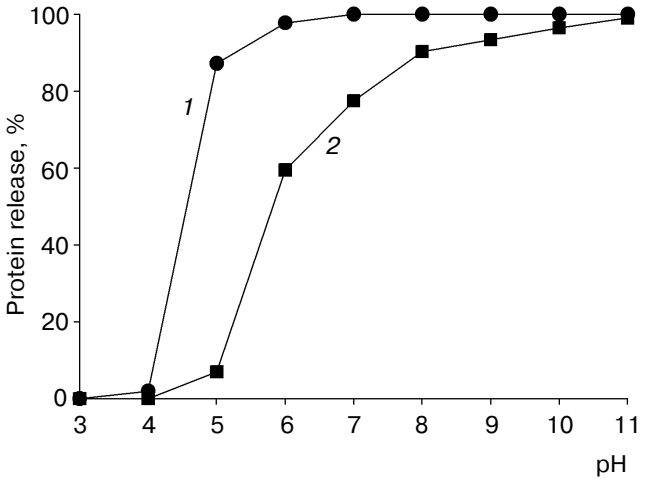

Fig. 3. Effect of pH on protein release from monolayer (*1*) and trilayer (*2*) microparticles. Incubation time 1 h.

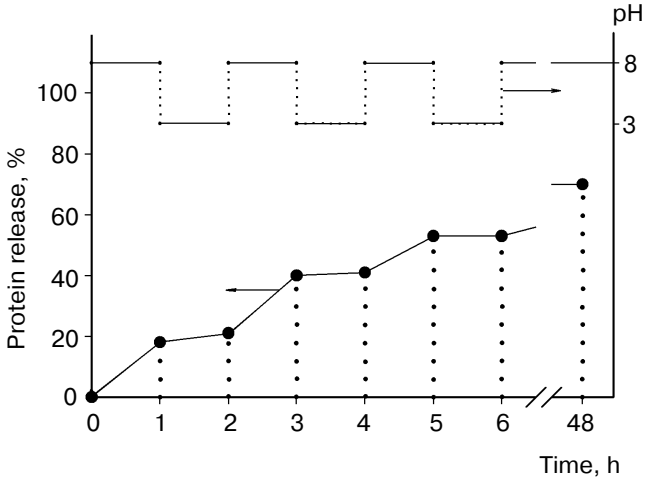

Fig. 4. Stepwise protein release from 11-layer microparticles on alternating pH change.

Steric hindrances for interaction between enzyme and substrate due to the complex formation with polyelectrolytes cause a decrease in enzymatic activity. As demonstrated by gel-permeation chromatography (Fig. 5b), on increase in the ionic strength from 0.2 to 2 M, ChT complex with polyelectrolyte decomposes almost completely, and enzymatic activity of protein released from trilayer microparticles increases 3.5-fold. Protein activity is thereby restored almost completely and is 73% of that of the native protein. It should be noted that ChT enzymatic activity after solubilization of protein aggregates is 86% of that of the native protein. On the whole, formation of enzyme complexes with polyelectrolytes and polyelectrolyte complexes can have no effect on enzyme activity or can significantly decrease this activity. This effect depends on the nature of the reactants and complex formation conditions [10-13].

Change in the ChT molecule conformation or complex formation with polyelectrolytes is another possible factor influencing activity of protein released from microparticles. Study of the second derivatives of UV adsorption spectra of the protein released from microparticles as a soluble complex with polyelectrolytes [14-16] showed a hypsochromic shift of λ_{max} for tryptophan and tyrosine residues in relation to the native ChT (Fig. 6). For tryptophan, λ_{max} changed from 291.2 (native protein) to 290.1 nm (protein in the complex), and for tyrosine residues λ_{max} changed from 283.7 to 282.2 nm, respectively.

The data indicate that the aromatic amino acid residues are more available to the polar solvent and that the native protein conformation changes significantly on the complex formation with polyelectrolytes.

At pH near the maximal enzymatic activity, autolysis typical of proteases drastically decreases the native protein activity. It is known that the complex formation of enzymes with polyelectrolytes can increase their stability

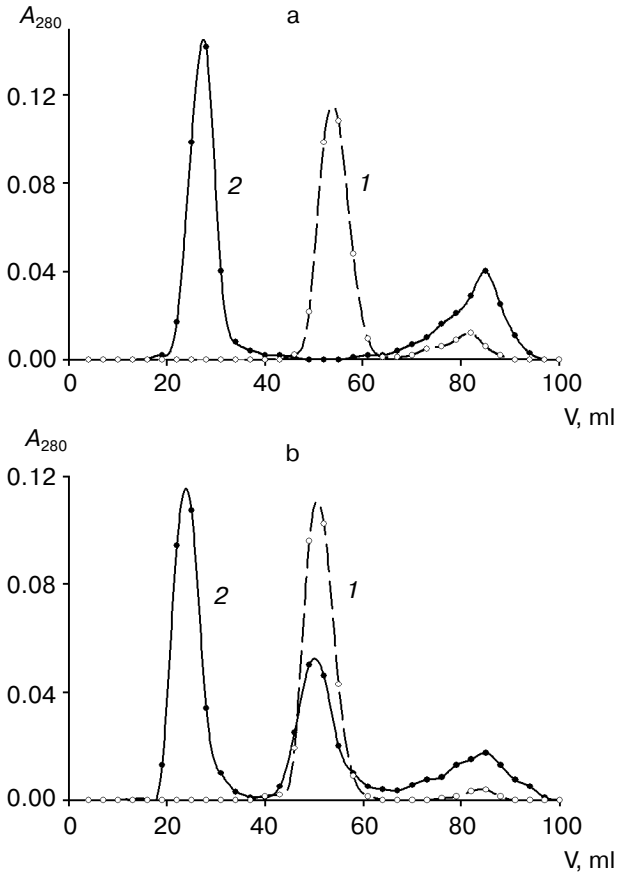

Fig. 5. Gel-permeation chromatography on Sephadex G-75 of protein released from trilayer microparticles at pH 6.0 (universal buffer, 0.2 M NaCl): native protein (*1*) and protein released from microparticles (*2*). Peaks on the right correspond to the products of ChT autolysis. Conditions: incubation time 2 h, 150 rpm; the ionic strength of the eluent corresponds to 0.2 (a) and 2.0 M (b) NaCl solution.

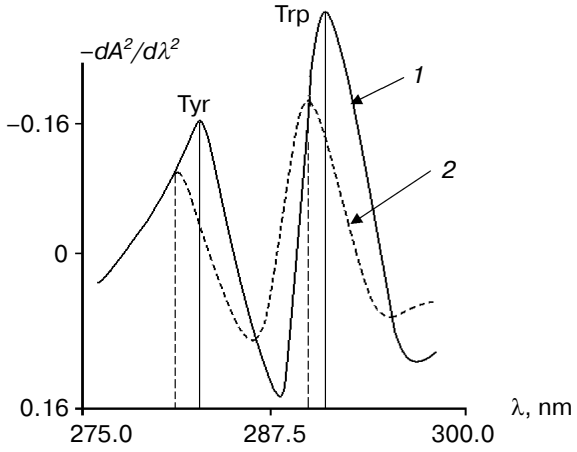

Fig. 6. Second derivatives of UV adsorption spectra of Tyr and Trp residues: native protein (*1*) and protein released from trilayer microparticles at pH 6.0 (*2*). Protein concentration 1 mg/ml.

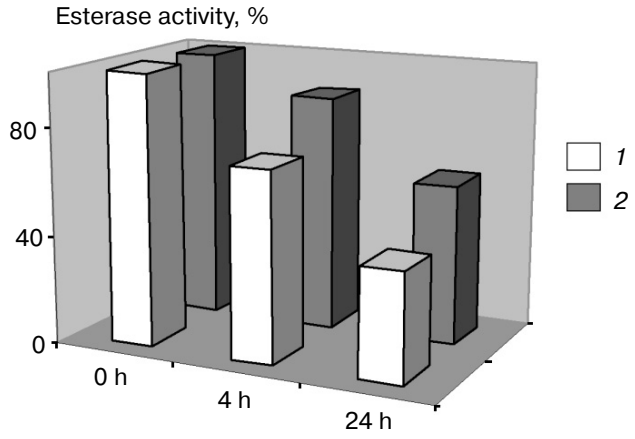

Fig. 7. Time-dependent change in esterase activity of native ChT (*1*) and protein released from trilayer polyelectrolyte microparticles (*2*). Conditions: pH 6.0 (universal buffer), 37°C.

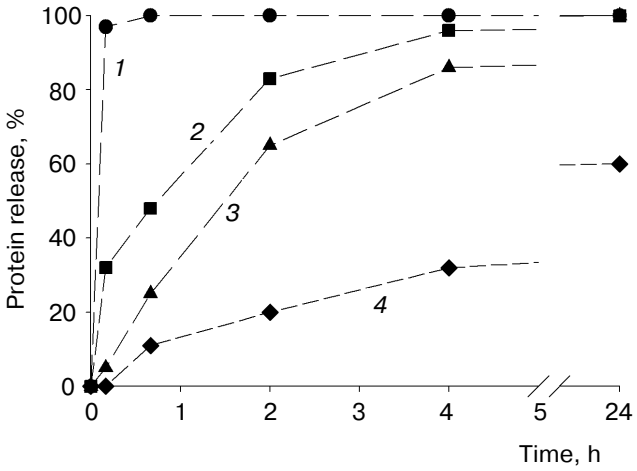

Fig. 8. Kinetics of ChT release from microparticles with various number of polyelectrolyte layers at pH 8.0 (Tris-buffer). Number of polyelectrolyte layers: *1*) 1; *2*) 3; *3*) 5; *4*) 11.

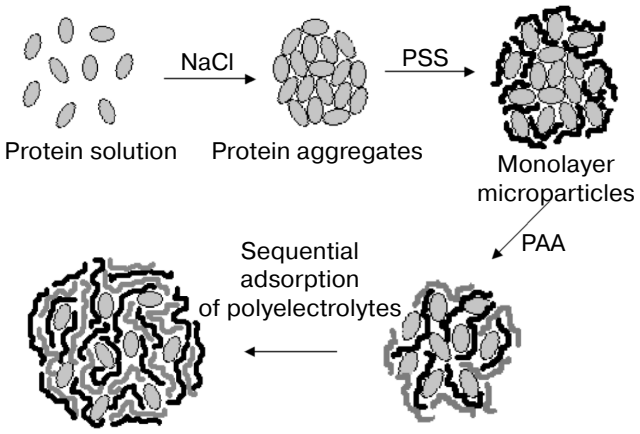

Fig. 9. Scheme of formation of ChT-containing polyelectrolyte microparticles.

to autolysis and denaturing conditions [17, 18]. Stabilization of ChT released from microparticles in relation to autolysis due to the complex formation with polyelectrolytes is an advantage of this immobilization method (Fig. 7). Thus, after incubation at pH 6.0 for a day at 37°C, the residual activity of the enzyme in the complex is almost 2 times higher than that of the native protein.

Kinetics of protein release from microparticles at pH 8.0 versus the number of applied polyelectrolyte layers is presented in Fig. 8. As shown, the initial rate of protein release from microparticles drastically decreases with increase in the number of polyelectrolyte layers from 1 to 11. Nonetheless, mono-, tri-, and pentalayer microparticles completely lose incorporated protein within several hours, whereas 11-layer microparticles hold almost half of the immobilized protein even after a day.

The dependence indirectly confirms that on alternative adsorption of the oppositely charged polyelectrolytes the structure of microparticles is stabilized by a net of polyelectrolyte complex, which inhibits protein release.

The process of formation of polyelectrolyte microparticles can be illustrated by the scheme shown in Fig. 9. On incubation of protein aggregates with PSS in solutions with high ionic strength their stabilization occurs; the latter results in formation of microparticles insoluble in solutions with low ionic strength. Monolayer

particles were thus obtained. Further sequential adsorption of polyelectrolytes results in incorporation of microparticles into a polyelectrolyte net that hinders diffusive protein release.

So, a new method of protein immobilization into polyelectrolyte microparticles by alternative adsorption of oppositely charged polyelectrolytes on the aggregates obtained by salting out of protein is proposed. Using ChT as an example, it is shown that this method allows protein incorporation into polyelectrolyte microparticles and obtaining of preparations stable on storage. Regulation of release of incorporated protein by change in pH and the number of polyelectrolyte layers is shown to be possible.

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