Immobilization of trypsin on polysaccharides upon intense mechanical treatment

A. N. Zelenetskii,[a] T. A. Akopova,[a]★ N. R. Kildeeva,[b] G. A. Vikhoreva,[b] E. S. Obolonkova,[a] and A. A. Zharov[c]

[a]N. S. Enikolopov Institute of Synthetic Polymer Materials, Russian Academy of Sciences, 70 ul. Profsoyuznaya, 117393 Moscow, Russian Federation. Fax: (095) 420 2229. E-mail: anzel@ispm.ru
[b]A. N. Kosygin Moscow State Textile University, 1 ul. Malaya Kaluzhskaya, 117918 Moscow, Russian Federation. Fax: (095) 952 1440. E-mail: vihorevag@mail.ru
[c]N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 47 Leninsky prosp., 119991 Moscow, Russian Federation. Fax: (095) 135 5328

Immobilization of a ptoteolytic enzyme, trypsin, on chitosan and cellulose was accomplished in the solid state under pressure and shear strain. Trypsin immobilized in this way retains 70—80% of the catalytic activity and exhibits prolonged action. The mechanical treatment allows introduction of a much larger amount of the active protein into a polymer matrix than the equilibrium sorption from solution.

Key words: polysaccharides, trypsin, immobilization, pressure and shear strain, release kinetics.

The polysaccharides chitosan and cellulose are nontoxic, biocompatible and are traditionally used in food, cosmetic and pharmaceutical industries as drug or bioactive substance carriers.[1]

The three-dimensional structure of proteins is exceptionally sensitive to changes in the medium pH, increase in temperature, and solvent effects. Usually, extreme treatment leads to unfolding of the polypeptide chain and, hence, to the loss of catalytic activity of the enzyme. However, immobilization of protein molecules onto a solid support considerably increases their stabilities. Moreover, immobilized enzymes can be easily removed from the reaction medium and reused. An easy way of incorporating enzymes into a polymer matrix is the formation of films from solutions (emulsions) containing both the polymer and the enzyme. However, these preparations do not provide a sufficiently prolonged action, which comes as their disadvantage. An alternative method for the immobilization of enzymes and drugs is blending with a polymer carrier in the solid state under mechanical treatment. This enables not only interfusion of the components at the molecular level but also chemical reactions in the absence of a liquid dispersion medium.[2] A distinctive feature of this method is the absence of a contact of enzymes with solvents, which induces denaturation.

It is known[3] that the molecules of enzymes consisting of a single polypeptide chain (*e.g.*, trypsin) usually acquire conformations close to a globule, which can also occur in the solid state, whereupon the chain segments that form the catalytically active site approach each other. In this conformation, hydrophobic segments of the macromolecule are plunged inside the globule to form a core stabilized by hydrophobic interactions, in addition to hydrogen bonds. The hydrophilic groups of side chains are located on the surface. The range of functional groups found in enzymes is extremely diverse; therefore, the interaction of the enzyme with polysaccharides containing polar groups can give rise to both hydrogen and chemical bonds. We have shown previously[4] that both chitosan salts and acylated derivatives of chitosan and cellulose can be prepared by application of pressure and shear strain in the solid state. Joint deformation of these polysaccharides under pressure resulted in changes in the structure of hydrogen bonds and chemical interactions between macromolecules.[5] The purpose of the present work is to study the feasibility of immobilization of trypsin owing to the formation of physical and chemical bonds between the protein molecules and polysaccharides upon joint deformation in the solid state and to select conditions for the mechanical treatment where the biological activity of the enzyme is retained as fully as possible.

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Experimental

The proteolytic enzyme, trypsin, with a molecular mass of 23800 D (Research and Production Association Biolar, Latvia) was used in the work. Wood cellulose (Bratsk Pulp-and-Paper Mill) and chitosan from crab shells (poly[(1→4)-2-amino-2-desoxy-β-D-glucose] containing 15% 2-acetamido-2-desoxy-D-glucose units, All-Russia Research Institute of Fishery and Oceanography) were mechanically pre-activated by treatment in a twin-screw extruder (Berstorff, Germany) for 3—5 min at ~20 °C. This type of treatment reduces the degree of crystallinity and the molecular mass of polysaccharides and increases the internal specific surface area.[6] The molecular masses of the resulting finely dispersed (~200 μm) cellulose and chitosan were 100 000 and 300 000 D, respectively. The molecular masses were determined from the intrinsic viscosity of solutions (a cuprammonium solution for cellulose[7] and an acetate buffer for chitosan[6]). The ash and moisture contents in the initial polysaccharides determined by conventional methods[7] were at most 0.15 and 5—7%, respectively.

Crystalline trypsin was mixed with polysaccharides at a mass ratio of 0.1 and treated in Bridgman anvils under a pressure of 1 GPa and a shear angle of 200°.

Reference samples were prepared by equilibrium sorption of the enzyme onto mechanically activated polysaccharides from an aqueous solution of trypsin with a concentration of 2 mg mL^{-1} at the same mass ratio (trypsin : polysaccharide = 0.1). The chosen concentration of the protein was optimal for maximum adsorption.[8]

The trypsin activity in the solution and in the immobilized state was determined based on the initial rate of hydrolysis of the substrate, namely, *N*-benzoyl-L-arginine methyl ester (BAME), at pH 8.0 on an autotitrator (Radiometer, Denmark) in the pH-stat mode. Hydrolysis of BAME catalyzed by the enzyme present in the cell ($V = 4$ ml) yielded an amino acid, which was automatically titrated with 0.01 *M* KOH at a rate necessary to maintain the pH at the specified level. The activity was expressed as the amount of substrate (μmols) hydrolyzed by 1 mg of the enzyme in 1 min at 20 °C. The error of determination of enzyme activity was 10%.

The protein concentration in the solution during sorption was analyzed according to Lowry.[9] The amount of enzyme having passed to the solution upon washing of immobilized trypsin samples was determined from analysis of the activity of washings using BAME.

The kinetics of release of immobilized trypsin from the matrix was measured in a physiological solution (water consumption in the bath, 10 mL g^{-1}) at ~20 °C. These desorption conditions mimic to an extent the behavior of protein compositions with polymer carriers in a living organism.

The microphotographs were obtained using a JSM-5300LV Jeol electron microscope.

Results and Discussion

Since an enzyme is active only as long as the specific tertiary structure of the protein is retained, it is necessary to find out what happens to this structure upon deformation under pressure. We assumed that study of the enzyme activity after solid-state deformation with polysaccharides and comparison of the results with the activity of the enzyme spread in the polysaccharide through equilibrium sorption from a solvent will provide valuable information on the retention of the protein tertiary structure under a powerful energetic action of pressure and shear.

Table 1. Change in the trypsin activity after mechanical treatment

Compound or blend	Activity*/units (mg of trypsin)$^{-1}$		Retention of activity (%)
	starting	after treatment	
Trypsin	37.5	22.0	58.7
Trypsin—cellulose	37.5	29.8	79.5
Trypsin—chitosan	37.5	26.8	71.5

* The number of μmols of the substrate hydrolyzed by 1 mg of the enzyme in 1 min at 20 °C is taken as the activity unit.

The results of measurements of the catalytic activity of native trypsin and its blends with cellulose and chitosan subjected to mechanical treatment are presented in Table 1. It can be seen that deformation of neat solid crystalline trypsin under these conditions decreases its activity by ~40%. As shown previously,[5,10] this can be due to destruction of hydrogen and ionic bonds that fix the native conformation of the protein, in particular, through interaction of protein molecules with each other. When cellulose or chitosan was used as the polymeric matrix for joint deformation with trypsin, ~80 and 70% of trypsin activity, respectively, was retained.

At the micrographs taken at ×5000 magnification, one can clearly see the characteristic crystal structure of the initial trypsin (Fig. 1, *a*). Deformation under pressure is known to lead to profound destruction of crystal structures down to ultramicrodisperse dimensions.[11,12] The obvious maintenance of the trypsin crystal structure after deformation (Fig. 1, *b*) must be related to recrystallization after pressure release. The presence of cellulose or chitosan during the joint deformation with trypsin may hamper self-association and recrystallization of the protein. After deformation of trypsin blends with either cellulose or chitosan (Fig. 1, *c* and *d*, respectively), no trypsin particles can be detected with the experimentally attained magnification.

The reference system we used was cellulose or chitosan pre-activated by mechanical treatment in an extruder and containing trypsin sorbed from a solution. According to theoretical views, this type of adsorption gives rise to molecular complexes stabilized by bonds of different nature and energy, namely, hydrogen, coordination, ionic, and covalent bonds, on the carrier surface. The experiment showed that the amount of protein that can be introduced

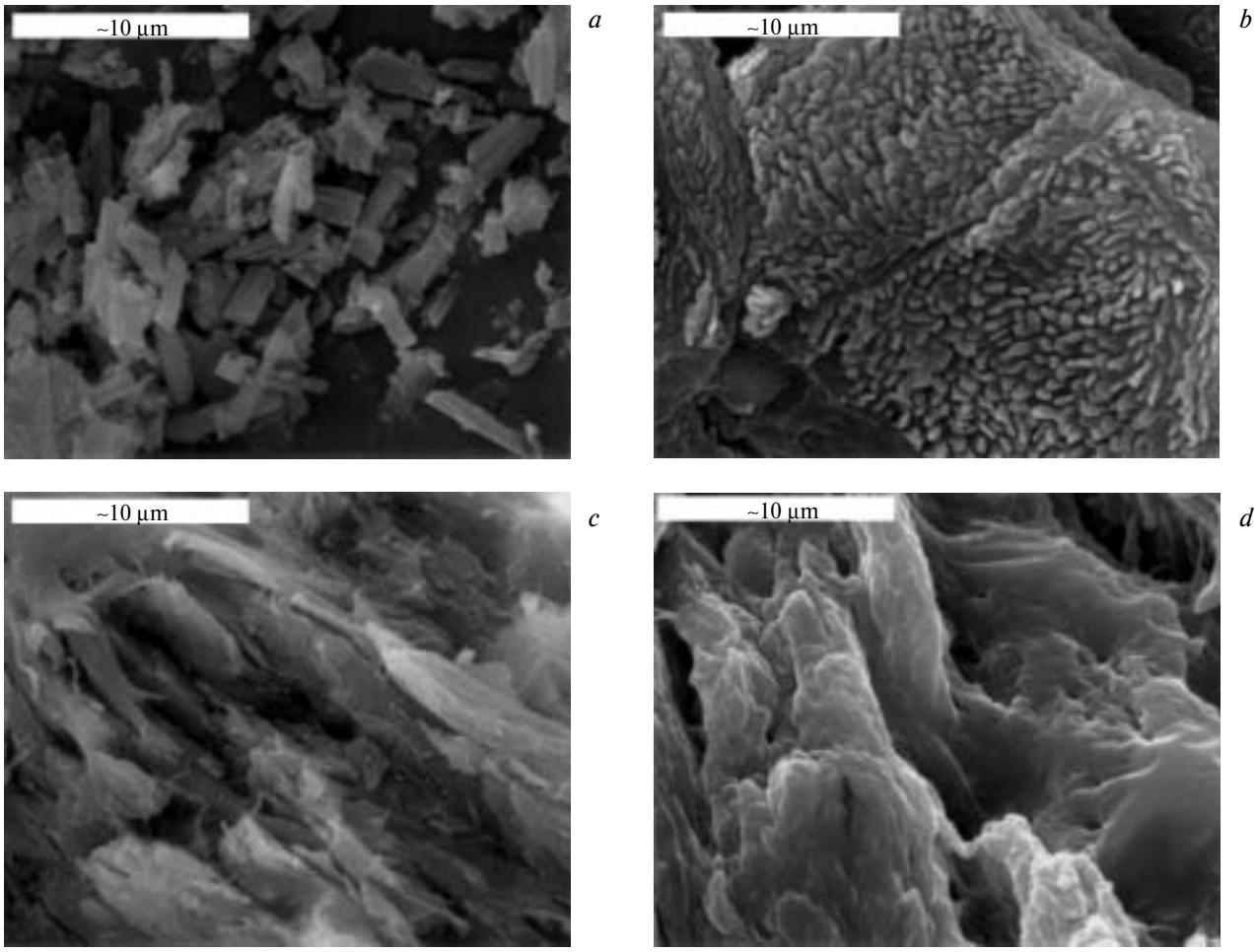

Fig. 1. Electron micrographs of initial trypsin samples (*a*) and samples after deformation in Bridgman anvils: trypsin (*b*), trypsin blends with cellulose (*c*) and chitosan (*d*).

by equilibrium sorption to polysaccharides at an enzyme : polysaccharide ratio of 0.1 is considerably lower (Table 2) than the amount introduced upon mechanical treatment. As a consequence, the activity of such samples is 6—8 times lower than the activity of samples obtained by solid-state synthesis. The absolute amount of trypsin bound irreversibly to the polymer carrier in the samples kept for 2 h in a physiological solution is also higher in the case of protein immobilization by joint solid-state deformation with polysaccharides. The ratio of the specific activities of the trypsin remaining after extraction in samples with the same carrier obtained by the two different methods (see Table 2) amounts to 1.6 for cellulose and 2.5 for chitosan. This implies that the mechanical introduction technique activates chitosan more appreciably than cellulose towards trypsin.

The kinetic data for trypsin desorption to a physiological solution indicate the possibility of prolonged action (up to 4 h) of the enzyme immobilized by both methods (Figs. 2 and 3), whereas trypsin incorporated in the polymer matrix during the formation of films from solutions of chitosan in acetic acid is fully released to the solution over a period of 30 min.[13]

Table 2. Comparative characteristics of trypsin immobilized on different polysaccharides by joint solid-state deformation under high pressure or by sorption from solutions by mechanically activated polymers

Polymer carrier	Enzyme content /mg (g of the carrier)$^{-1}$	Activity/units (g of the carrier)$^{-1}$	
		starting	after extraction*
	Joint deformation		
Cellulose	100	2980	328
Chitosan	100	2680	161
	Sorption from solution		
Cellulose	15	562	202
Chitosan	8	300	63

* For 2 h.

By investigating the pattern of enzyme release into a physiological solution, one can not only estimate its ac-

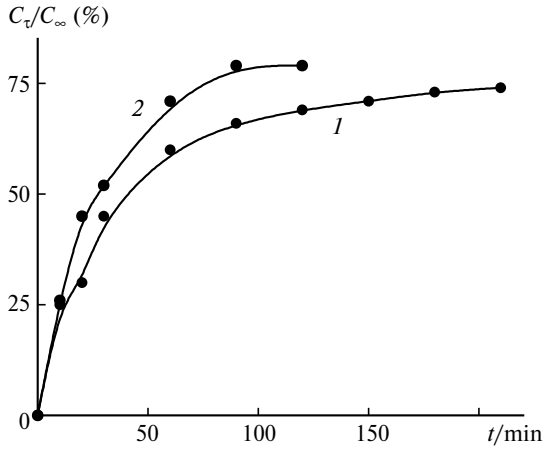

Fig. 2. Kinetic curves for trypsin release from samples obtained by adsorption of the enzyme on cellulose (*1*) and chitosan (*2*). C_τ is the current concentration of the enzyme released, C_∞ is the maximum possible concentration of the released enzyme.

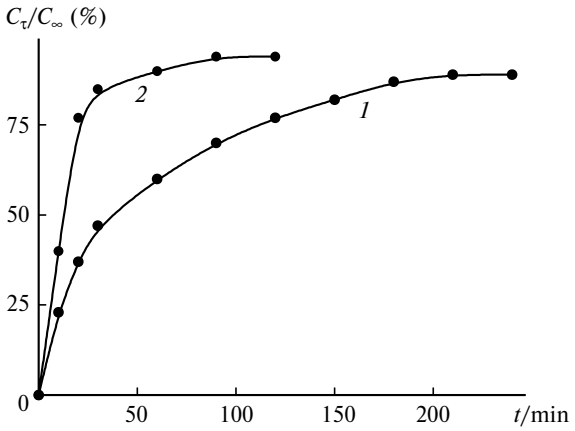

Fig. 3. Kinetic curves for trypsin release from samples obtained by joint solid-state deformation of the enzyme with cellulose (*1*) and chitosan (*2*). C_τ is the current concentration of the enzyme released, C_∞ is the maximum possible concentration of the released enzyme.

tivity *in vivo* but also draw a substantiated conclusion concerning the distribution and the binding energies of protein molecules in the polysaccharide matrix. The ratio of rates of protein release from cellulose and chitosan is the same for the reference sample obtained by sorption and the solid-state sample, in particular, in both experiments, at the initial stage, chitosan holds the enzyme less strongly than cellulose, the curve of trypsin desorption from chitosan reaching earlier its limit. However, the relative amounts of protein remaining on the carrier in the final stage of desorption are almost the same for cellulose and chitosan, irrespective of the way of formation of intermolecular bonds (see Fig. 2, 3). It can be concluded that the protein is bound to the polysaccharide by adsorption forces of the same nature in both the sorptional and solid-state methods. However, the number of intermolecular bonds and, hence, the number of protein molecules introduced is considerably higher for the solid-state method, and the energy spectrum of these bonds is much broader. A similar change in the spectrum of intermolecular interactions due to hydrogen bonds and the formation of ionic structures upon proton transfer has been observed for joint deformation of a great number of amines and organic acids or alcohols under pressure.[14] The broad energy distribution of the hydrogen bonds between donors and acceptors formed after deformation was responsible for a larger number of IR absorption frequencies compared to the hydrogen bonds formed in solution. The complete proton transfer was observed for donor—acceptor pairs that do not form salts in solutions.

The larger variety of bonds in the product of solid-state synthesis accounts for the faster release of the protein. Indeed, the weakest intermolecular bonds are cleaved at the beginning of desorption, while a considerable number of protein molecules fixed by stronger intermolecular interactions, formed during mechanical dispersion of the protein in the matrix, are retained on the carrier in the final stage.

Thus, we have shown that the action of high pressure and shear strain gives rise to stable bonds between trypsin and the polysaccharide matrix. This enables the introduction of a substantial amount of the enzyme without using liquid media. Both trypsin and chitosan possess wound-healing properties, while cellulose is traditionally used as a bandaging material; therefore the resulting compositions of trypsin with polymer carriers, which exhibit high catalytic activities and prolonged action of the immobilized trypsin, can be successfully used in medicine.

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