A New Biosynthetic Material and Its Potential Application Domains

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ABSTRACT: A polysaccharide was obtained by fermentation of glycerol in the presence of *Pseudomonas* spp. bacteria. It was characterized by FTIR spectroscopy, SEC chromatography, conductimetric titrations, and viscometric measurements; its emulsifying activity was tested using various mixtures of a hydrocarbon compound and polymer solution. Based on this polysaccharide, new crosslinked ionic derivatives were synthesized and characterized; their interaction with lysozyme was studied. From the data presented, one can suggest some applications for this new, less expensive polysaccharide—it could be used as a thickener and specific bioemulsifier, while its derivatives—as a support for controlled release of biomolecules. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 159–167, 2011

Key words: pseudozan; polysaccharide; lysozyme; controlled release

INTRODUCTION

In recent years, polysaccharides have received great attention as an alternative to synthetic polymers, due to the multitude of their application domains, correlated with their biocompatibility, biodegradability, and lack of toxicity—they can be used as plasma expanders (dextran), bioemulsifiers,[1] and rheothickeners.[2] The presence of (hydroxylic, amine, carboxylic) reactive groups on the macromolecular chains allows numerous chemical modifications leading to a wide range of polysaccharide derivatives, with applications in the food, cosmetic, pharmaceutical industries, or in biotechnology. The potential of water-soluble polysaccharides to impart particular properties to solutions and gels is especially significant when it comes to applications involving issues of environmental impact, biocompatibility, and/or biodegradability. For specific applications, it is necessary to optimize the final properties by controlling the chemical structure and/or physical properties. Research in the field of polysaccharides cannot be practically exhausted, many derivatives, chemical transformations, and application domains being still insufficiently explored.

Polysaccharide hydrogels represent a very important type of natural polymers, with a multitude of applications in the biomedical, biotechnological, and pharmaceutical fields; they represent the polymer network physically[3] or chemically[4] crosslinked, able to retain high water amounts. By the variation of the polysaccharide support and by chemical transformation of the functional groups, a multitude of derivatives can be obtained.

Glycerol, a by-product from the biodiesel industry, has proven to be a suitable carbon substrate for polysaccharide production, its use drawing significant cost reduction. Freitas et al. carried out extensive work on the synthesis, characterization, and potential application domains of a polysaccharide obtained from glycerol.[5–7]

Our study focuses on the synthesis and characterization of a glycerol-based polysaccharide (Pseudozan, abbreviated: Psz) obtained in the presence of *Pseudomonas* spp.; the derivatives of this polysaccharide, which, to our knowledge, have not been reported yet, may have interesting specific properties. The paper also presents the synthesis of some crosslinked derivatives of pseudozan and evaluates their interaction with biomolecules, with the aim of determining new potential application domains.

MATERIALS AND METHODS

Materials

Pseudozan

Pseudozan was produced by the aerobic fermentation (at 30°C) of a naturally isolated bacterial strain of *Pseudomonas* spp., in a cultivation medium

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containing glycerol (by-product from the biodiesel industry) as a carbon source substrate, corn steep liquor as a nitrogen source, and salts. The new pseudozan-producing *Pseudomonas* spp. strain was isolated in the laboratory from a sample of oil-contaminated soil and—designated as *Pseudomonas* spp. ICCF-400—it was stored as a part of the INCDCF collection of microorganisms (WDCM232). It was taxonomically affiliated by biochemical and morphological tests using a biological microbial identification system—GN_2 Microplate (Biolog Inc., Hayward CA) and Bergey's Manual of Determinative Bacteriology.[8]

From 35 g/L of glycerol, 20–24 g/L of crude polysaccharide were obtained after 50–55 h of fermentation. The fermentation broth reached an apparent viscosity of 200–230 mPa.s (120 s^{-1}). The downstream processing included: isolating the crude product by ethanol precipitation and drying, suspending it in water, separating the nonsoluble fraction by filtration, purification and concentration of the polysaccharide from the filtrate by ultrafiltration-diafiltration. From the final concentrate, pseudozan was isolated by ethanol precipitation, it was separated, and then dried under vacuum at 85°C.

Crosslinked pseudozan microparticles

Crosslinked pseudozan microparticles were synthesized by dispersion of an aqueous alkaline solution in an organic suspension medium, using cellulose acetobutyrate as stabilizer and epichlorohydrin as crosslinker, as previously described for polysaccharide pullulan.[9] Briefly, 30 mL of 20 g % pseudozan alkaline solution were added to 100 mL of dichloroethane containing 5 g of cellulose acetate butyrate; after a 1 h dispersion at 50°C, 3 mL of epichlorohydrin were added and the reaction was allowed to proceed for 24 h at 50°C; then the microparticles obtained were filtered, washed with acetone and water, and subsequently dehydrated from methyl alcohol; yield: 6.0 g (~ 84%).

Functional derivatives

Functional derivatives with anionic carboxymethyl (CM) or sulfopropyl (SP) groups were synthesized as previously described for curdlan-based products.[10]

Carboxymethylation of Psz microspheres (CM-Psz)

1 g of Psz microspheres was suspended in 5 mL of isopropanol, and swollen with a 5 mL 10% (w/v) NaOH solution; then 2.16 g (18.5 mmoles) of sodium chloroacetate were added to 4 mL of water and the reaction was allowed to proceed for 5 h at 70°C. The carboxymethylated microparticles were filtered, washed with water, and dehydrated from methyl alcohol; yield: 1.10 g (~ 75%).

Sulfopropylation of Psz microspheres (SP-Psz)

1 g of Psz microspheres was suspended in 5 mL of isopropanol and the microspheres were allowed to swallow in 5 mL of 10% (w/v) NaOH solution; 2.26 g (18.5 mmoles) of propane sultone were added and the reaction was allowed to proceed for 6 h at 50°C. The sulfopropylated microparticles were filtered, washed with water, and then dehydrated from methyl alcohol; yield: 1.25 g (~ 87%).

Carboxymethylation/Sulfopropylation of Psz microspheres (CM/SP)

To obtain both weak and strong acidic anionic groups on the same chain, the Psz microspheres were chemically modified by introducing carboxymethyl and sulfopropyl groups; the reaction was carried out on 1 g of Psz microspheres, suspended in 5 mL of isopropanol, swollen in 5 mL of 10% (w/v) NaOH solution. 1.08 g (9.3 mmoles) of sodium chloroacetate dissolved in 2 mL water and 1.13 g (9.3 mmoles) propane sultone, divided into portions, were added alternatively. The reaction was allowed to proceed for 5 h at 60°C. The microparticles obtained were filtered, washed with water, and then dehydrated from methyl alcohol; yield: 1.30 g (~ 77%).

Other materials

Span 80 (Fluka); lysozyme (Fluka Chemie Gmbh, packed in Switzerland), $M_w \sim 14600$ g/mol.

Methods

The physicochemical characterization of the polysaccharide was performed through:

Elemental analyses [EDAX—Environmental Scanning Electron Microscope (ESEM) type Quanta 200]

The carbon content was determined as varying between 54 and 78% (w/w); the elements identified—K, Mg, Ca, S, Si, K, Na—were found in variable proportions, ranging between 0 and 1% (w/w).

FTIR

FTIR spectra were recorded in transmission on a Bruker Vertex 70 spectrophotometer.

Conductimetric titrations

Conductimetric titrations were performed using a Mettler Toledo SevenEasy™ S30 conductometer.

Steric exclusion chromatography

The chromatographic system was a HPLC Shimadzu (USA) with a refraction index detector, using a gel permeation column (250 mm × 4.6 mm i.d., 5 μ particle size, 300 Å pore size, Grace Davison Discovery Science, US). The mobile phase was 1*N* NaOH, at a flow rate of 1 mL/min. Standard samples of dextran (MW: 50,000, 80,000, 150,000, 270,000, 410,000, 670,000) purchased from Sigma were used.

Molecular parameters of pseudozan

Peak#	M_n	M_w	M_z	M_{z1}	M_v	M_w/M_n	M_v/M_n	M_z/M_w	I.Visc	%
Total	160,661	823,675	2,306,045	4,025,518	0	5.1268	0	2.7997	1	100

Viscometric measurements

Viscometric measurements were performed using a Ubbelhode capillary viscometer (0a, $k = 0.005094$), at 20°C, in aqueous or NaCl solutions.

Emulsifying activity

Emulsifying activity was determined using water-organic solvent mixtures, at various concentrations of the polysaccharide added and was evaluated by the emulsifying index:

$$\text{Emulsifying index} = \frac{\text{emulsion\_volume}}{\text{total\_volume(water + hydrocarbon\_compound)}} \times 100\%$$

A method described by Cooper[11] was used: 5 mL of organic compound was mixed with 5 mL of aqueous polysaccharide solution in a test tube (20 mm diameter/200 mm length) and stirred in the wortex at 2400 rpm for 2 min. After 24 h, the emulsifying index was determined; all tests were performed in triplicate.

Interaction of microparticles with biomolecules

Lysozyme (a globular, low molecular weight, basic enzyme with antimicrobial activity) was used to evaluate the performance of the supports obtained for controlled drug delivery. The retention of lysozyme was studied under "batch" conditions, in glass-stoppered flasks, at 20°C; lysozyme deionized water solutions of known concentration were added to 50 mg of dry support, in the presence of sodium azide as a preservative (0.025% w/v); aliquots were withdrawn and the protein concentration in the supernatant was determined according to the modified Folin method,[12] on a Specord 200 Analytic Yena UV-vis spectrophotometer. The amount of retained protein was calculated as a difference between the initial protein content of the solution used and the protein content determined after retention. The lysozyme release was observed in solutions simulating gastric (pH 1.2) and intestinal (pH 7.2) fluid, on 50 mg samples, under "batch" conditions; aliquots of the solution were withdrawn and the protein content was determined again by the Folin method; the volume withdrawn was replaced with fresh releasing media. All measurements were carried out in triplicate. The release kinetics was calculated as: C_t/C_T, where: C_t is the amount of the lysozyme at a given time and C_T is the total amount of lysozyme.

The solution regain

The solution regain (g solution/g dry microspheres) was determined by centrifugating the microspheres swollen for 24 h up to reaching equilibrium (centrifuge: Janetzki T23, Poland) by Pepper's method.[13]

Pore volume

Pore volume (Vp) of the microparticles was determined from cyclohexane retention.[14]

Ionic group content

Ionic group content (meq/g) of Psz microparticles was determined through conductimetric titrations (COOH groups) and sulfur analyses based on Schöniger's method[15] (sulfopropyl groups).

RESULTS AND DISCUSSION

Linear pseudozan

The polysaccharides produced by *Pseudomonas* spp. grown on glycerol products were composed of

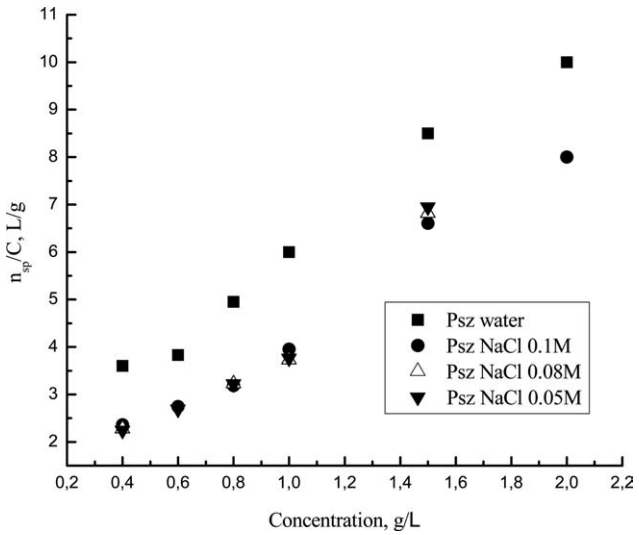

Figure 1 Specific viscosities of pseudozan, as concentration function in aqueous and NaCl 0.1*N* solutions.

neutral sugars: galactose, glucose, rhamanose, fucose, and acyl groups[5]; further studies are in progress aiming at determining the main constituent sugar residues of the polysaccharide synthesized under the selected set of conditions.

FTIR

FTIR spectrum presents bands around 3500 cm^{-1}, 2980 cm^{-1}, and 1100 cm^{-1}, characteristic of —OH; —CH; —CH_2 groups of the polysaccharides; a band around 1660 cm^{-1}, which can be attributed to ring stretching of galactose and manose[16]; this band may also have a contribution to COO^- asymmetric stretching[17]; the bands at 1720 cm^{-1} and 1403 cm^{-1} (symmetric stretching) also suggest the presence of COO^- groups.

Conductimetric titrations

Conductimetric titrations of pseudozan evidenced the presence of carboxylic groups in an amount of about 0.8 meq/g. Due to the presence of these charged anionic groups, the polysaccharide presents polyelectrolyte behavior in solution.

GPC

GPC measurements were used to determine the molecular parameters of pseudozan presented in the above given table: Molecular parameters of pseudozan; thus, M_w, $\overline{M}_n$, and PD (polydispersity index) values of about 824,000, 161,000, and 5.1, respectively, were obtained.

Viscometric measurements

Viscometric measurements revealed higher specific viscosity values of polysaccharide aqueous solutions, as compared to NaCl solutions, confirming the polyelectrolyte character of pseudozan (Fig. 1). This behavior can be explained by the screening ionic forces in NaCl solutions, which lead to the reduction of the macromolecular chain dimensions.

The intrinsic viscosities can be obtained by extrapolating specific viscosities to an infinite dilution, using the Huggins equation:

$$\eta_{sp}/C = [\eta] + k_H[\eta]^2$$

where C—solution concentration, k_H—Huggins constant, which depends on the nature of polymer/polymer interactions in solution and provides information on the interactions between the polymer chain and the solvent used. Values ranging between 0.3 and 0.8 are attributed to a random coil polymer conformation in a good solvent, while values higher than 0.8 correspond to a rigid polymer backbone and may result from intermolecular associations. The high values of Huggins constants (Table I) can be attributed to some branching on the polysaccharide backbone, which may determine intermolecular associations; this aspect will be verified in further studies. The Huggins constants of pseudozan in NaCl solution are higher than those determined in an aqueous solution, which correlates with the decrease in intrinsic viscosity values in this medium. The same variation of Huggins constants and intrinsic viscosities in water and NaCl solutions was obtained for the polysaccharide obtained from glycerol by Hilliou et al.[18]

The transition from dilute to semidilute solution ($C_{crt.}$) can be observed by plotting the viscometric data in bi-logarithmic coordinates, by the Utracki-Shima representation.[19] The $C_{crt.}$ can be considered as a measurement of the degree of space occupancy of the polysaccharide. The break of the plots corresponds to the transition between dilute and semidilute solutions and therefore to the beginning of chain entanglement. The data for the studied polysaccharide are presented in Figure 2. As can be seen, $C_{crt.}$ for NaCl 0.1*N* solution (0.8 g/L) is higher than that for the aqueous solution (0.6 g/L).

The higher $C_{crt.}$ value of NaCl solution than that of the aqueous solution (Fig. 2) may be explained by the more compact structure of the polymer with anionic groups screened by NaCl, which induces a

TABLE I
Pseudozan Intrinsic Viscosities and Huggins Constants in Aqueous and NaCl 0.1*M* Solutions

Pseudozan solution	[η]	k_H
Aqueous	3.2	0.77
NaCl 0.1*M*	2.09	1.73

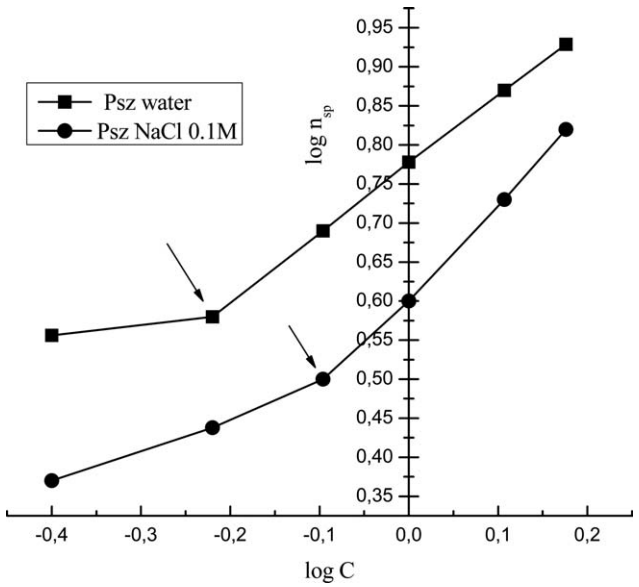

Figure 2 Bi-logarithmic plotting of ς_{sp} versus polymer concentration.

reduced hydrodynamic volume, hence, higher concentrations seem necessary to occupy the same space. Intermolecular associations are favored, allowing an easier packing of the space—this property recommends polymers for their use as thickeners even in saline media.

Emulsifying behavior

Many microbial polymers are reported to be useful as bioemulsifiers due to their ability to stabilize emulsions between water and hydrocarbon compounds. Comparatively with chemically synthesized emulsifiers, which are used in the food, pharmaceutical, cosmetic, and petroleum industries, emulsifiers from microbial sources are more advantageous due to their lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, higher selectivity, and specific activity at extreme temperature, pH, salinity, and due to their availability through synthesis from renewable feed stocks.[20]

Pseudozan was tested as a bioemulsifier to determine its capacity to stabilize hydrocarbon/water emulsions. Various organic compounds were used to study the emulsion-stabilizing capacity of the polysaccharide (mineral oil, *n*-hexane, xylene, benzene, chloroform), as a function of concentration. The experiments were performed at room temperature. The results presented in Figure 3 show that, at 0.5% (w/v) polymer concentration, only 3 substances (mineral oil, chloroform, and benzene) fulfilled the criterion for the emulsion-stabilizing capacity proposed by Willumsen and Karlson,[21] i.e., the ability to maintain at least 50% of the original emulsion volume 24 h after its formation. When increasing the

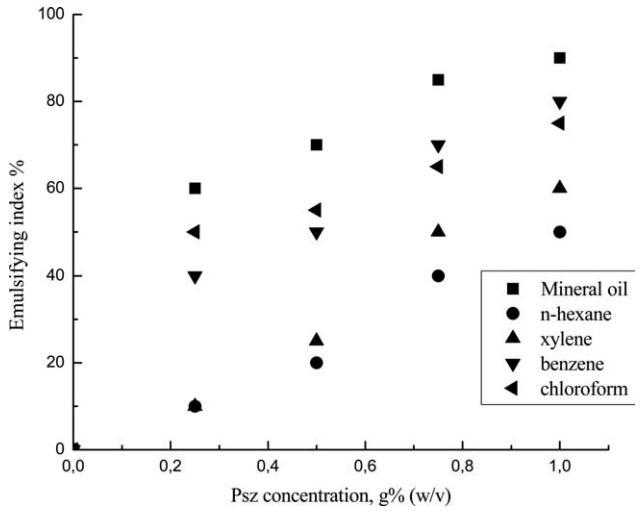

Figure 3 Emulsifying index as function of Psz concentration.

polymer concentration up to 1% (w/v), the emulsifying index reached or even exceeded 50% for all the organic compounds studied.

The emulsifying activity of pseudozan was also evaluated in comparison with a chemical surfactant, Span 80; the experiments were run at room temperature. The results presented in Figure 4 show that at the concentration used [0.25 g % (w/v)] only mineral oil and chloroform reach an emulsifying index of 60% or 50%, as compared to 50–50% presented by Span 80.

The testing of pseudozan's emulsifying behavior shows that this polysaccharide presents an improved emulsion-stabilizing capacity specific to certain hydrocarbon compounds; a similar behavior has

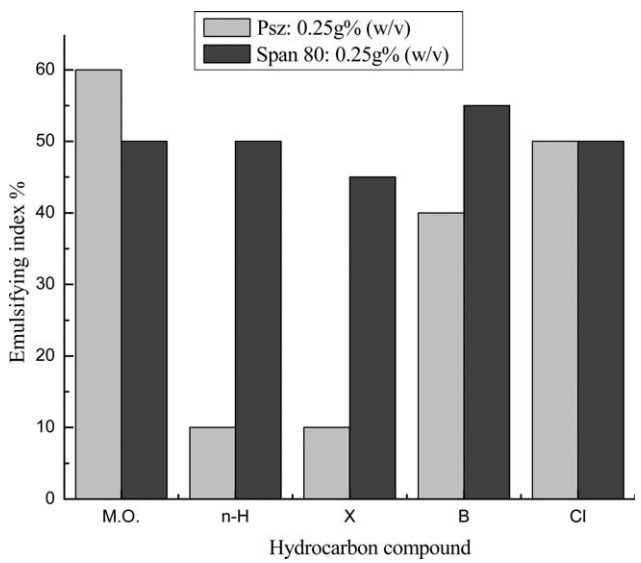

Figure 4 Emulsifying index of an aqueous Psz 0.25 g % (w/v) solution, comparatively with 0.25 g % (w/v) Span 80.

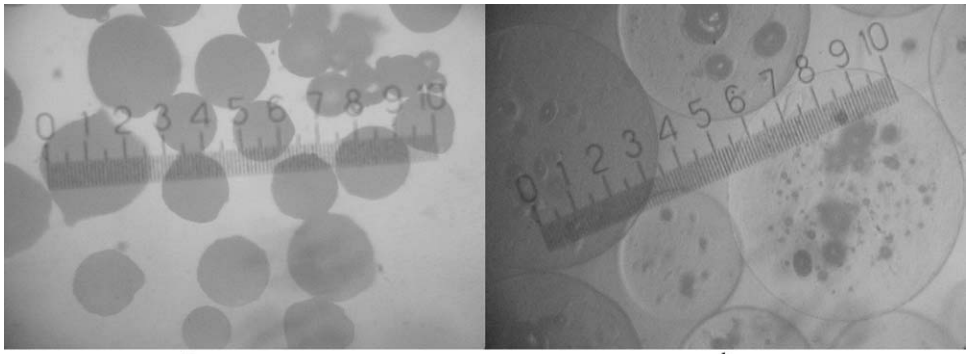

Figure 5 Photos of crosslinked pseudozan microparticles in dry (a) and swollen (b) state.

been reported for other microbial bioemulsifiers.[22,23] Further studies are in progress seeking to evaluate the influence of temperature, salinity, and pH on the emulsifying behavior of the new synthesized polysaccharide.

Crosslinked pseudozan microparticles

Physicochemical characterization of pseudozan microparticles

Polysaccharide hydrogel microparticles have been studied extensively due to their potential applications in biomedical, pharmaceutical, biotechnological domains—they can be used as supports for controlled drug release,[24] as delivery systems for pharmaceutically active peptides, proteins.[25–27] For this reason, we synthesized and characterized crosslinked hydrogel microparticles based on the new, less expensive polysaccharide pseudozan. Also, we studied their interaction with proteins, such as lysozyme, to evaluate their performance as supports for controlled release of biologically active molecules. Photos of dry and swollen pseudozan microparticles are presented in Figure 5.

Table II presents the physicochemical characteristics of crosslinked hydrogel microparticles based on pseudozan.

As may be observed, by the introduction of additional functional groups (besides the carboxylic ones contained in initial pseudozan), the water uptake of the microparticles (hence their hydrophilicity) increases; this water swelling is explained by the repulsion among the charged groups fixed on the macromolecular network. The water uptake of the supports with additional sulfopropyl groups (SP-Psz, SP/CM-Psz) is higher than that of the supports containing only COOH groups, due probably to their higher acidity. The degree of swelling decreases with an increasing ionic strength (uptake of 0.1*M* NaCl, pH 7.2) because of a diminishing repulsion. The swelling of supports containing ionic groups is also pH dependent: the repulsion among charged groups is maximum at pH values where the ionic groups are fully charged and decreases as the charged groups become neutralized; thus, the supports containing only —COOH groups have the smallest uptake of pH 1.2 solution, while for the supports containing sulfopropyl groups, besides —COOH, the shrinking of solution uptake at pH 1.2

TABLE II
Physico–Chemical Characteristics of Pseudozan-Based Crosslinked Pseudozan Microparticles

Sample	Functional group	Ionic group content (meq/g)	Pore volume (mL/g)	Solution uptake (g/g)			
				Water	NaCl 0.1*M*	pH 1.2	pH 7.2
Psz-C	—COOH groups from Psz	0.8[a]	0.19	8.0	7.83	5.60	7.90
CM-Psz	$-CH_2COOH$[a] (carboxymethyl)	2.05[a]	0.2	14.03	9.51	6.31	9.88
	—COOH[a] groups from Psz						
SP-Psz	$-(CH_2)_3SO_3H$ (sulfopropyl)	0.46[b]	0.23	17.08	9.33	7.56	9.86
	—COOH[a] groups from Psz	0.8[a]					
SP/CM-Psz	$-(CH_2)_3SO_3H$	0.46[b]	0.23	17.80	10.50	7.73	11.50
	$-CH_2COOH$	1.50[a]					
	—COOH groups from Psz						

[a] Determined from conductimetric titrations.
[b] Determined from sulfur analyses.

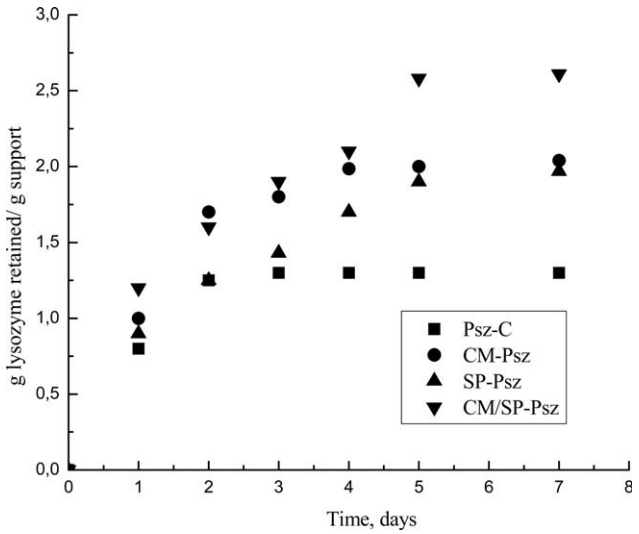

Figure 6 Retention curves of lysozyme on crosslinked Psz microparticles.

is less pronounced. The swelling properties of the support can influence its behavior as to the retention/release of biomolecules. Pore volume in a dry state is almost insignificant, but in a swollen state it becomes notable, the water uptake being a measure of the network hydrophilicity.

Interaction of crosslinked pseudozan microparticles with biomolecules

Retention of lysozyme

Lysozyme was loaded onto the supports from its aqueous solution, which is in accordance with the literature data reported for lysozyme retention on SP Sepharose[28,29] (higher lysozyme uptake capacities with decreasing ionic strength) and with our data obtained for other cationic polysaccharide supports[9] (retention of lysozyme from aqueous solutions; lysozyme release increases with the increase of the ionic strength of the salt solution, due to the screening of the electrostatic attraction between the two ionic species by the added salt[30]).

Lysozyme retention on the studied supports, preponderantly due to electrostatic forces, occurs through the formation of a polyelectrolyte complex: basic enzyme-anionic polysaccharide. The amount of lysozyme retained on pseudozan microparticles depends on the presence and nature of the functional group, as well as on the water uptake of the supports, which ensure the access of the protein into the polysaccharide tridimensional network. Thus, as may be remarked in the plots presented in Figure 6, the microparticles based on initial pseudozan, with low content of ionic groups and lower water uptake, retain a smaller amount of lysozyme than their derivatives with additional anionic functional groups and higher hydrophilicity.

The retention process of the biomolecule on pseudozan microparticles is influenced both by their anionic charges, which favor the electrostatic interactions with the basic lysozyme, and by the controlled diffusion of the enzyme within the swollen polysaccharide network. The highest lysozyme amount retained was obtained on the Psz derivative, the most highly charged with both weak and strong acidic anionic groups: sulfopropyl and carboxymethyl (SP/CM Psz); CM-Psz, having a higher content of anionic groups, retains more lysozyme than SP-Psz, which has fewer ionic charges, even sulfopropyl groups are strongly acidic, as compared to the carboxymethyl ones, which are weakly acidic.

In vitro release of lysozyme

Generally, the literature data present the release of the lysozyme retained on the supports using solutions with various ionic strengths.[31,10] The inclusion of biomolecules into macromolecular supports by chemical or physical methods ensures the controlled release of biomolecules as a function of pH and duration, in addition to a higher chemical stability against external factors. Release studies were performed with the aim of assessing the performance of the newly synthesized supports in controlled drug delivery; for this purpose, we used solutions simulating gastric fluid (pH 1.2) and intestinal fluid (pH 7.2); both pH values are below the isoelectric point of lysozyme ($\sim$ 11), when it has a net positive charge.

In acidic pH 1.2 solutions, the carboxylic groups of the supports turn to their uncharged form (COOH), thus releasing the lysozyme retained through electrostatic forces; the pseudozan microparticles with a lower content of ionic groups release the enzyme faster, even if its diffusion in solution may be hindered by the lower hydrophilicity of the less swollen microparticles. For the supports containing weakly acidic COOH groups and more highly acidic sulfopropyl groups (which are ionized over a very wide pH range), the lysozyme release in acidic solution is a resultant of the effects of these groups. A slower lysozyme release rate was shown by the support containing the highest amount of charged groups (SP/CM-Psz) [Fig. 7(a)]. It is worth mentioning that in acidic pH, despite the higher number of collapsed microparticles, the release occurs with a "burst" effect, for all supports—this behavior can be explained by the higher solubility of the lysozyme at this pH. The lysozyme is released gradually and one can suppose that the polysaccharide network can

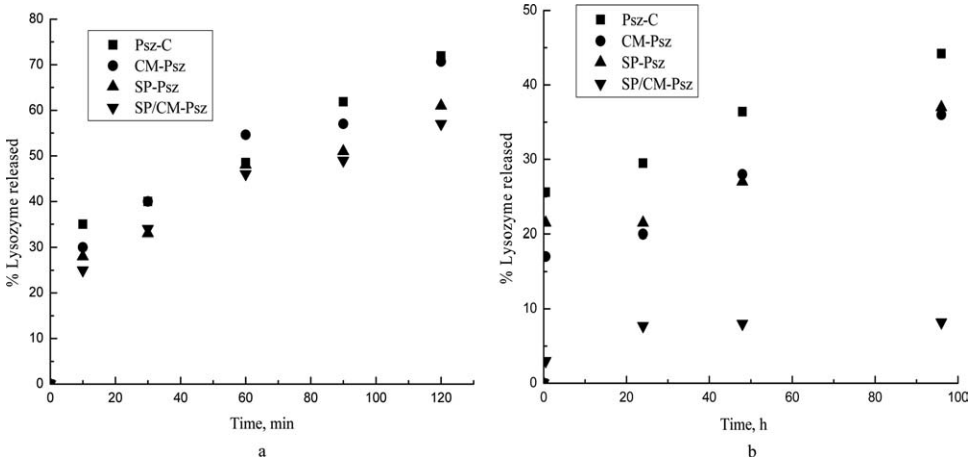

Figure 7 *In vitro* release curves of lysozyme retained on Psz microparticles in acidic (pH 1.2) (a) and buffered medium (pH 7.2) (b).

assure enzyme protection against the acidic fluid, during its passage through the stomach (~ 2 h).

In a solution simulating intestinal fluid (pH 7.2), the release of lysozyme from all supports occurs at a lower rate than in the gastric one, the lowest rate being recorded for the most charged pseudozan derivative—SP/CM-Psz [Fig. 7(b)]. Taking into account the fact that the main adsorption of the drugs occurs in the intestine, this controlled release can be beneficial.

The release behavior of the studied enzyme is influenced both by the content of ionic groups linked on the macromolecular network (which controls the chemical stability of the polysaccharide-lysozyme complex) and by the pH of the medium (which influences both the solubility of the biomolecule and the shrinking/swelling of the ionic network). Hence, by an appropriate choice of the support, one can control the retention/release of the biomolecule on/from the synthesized derivatives.

CONCLUSIONS

Previous characterization studies of pseudozan, revealed interesting physicochemical properties that recommend it for its use as a thickener or as a specific bioemulsifier.

Based on this polysaccharide, new ionic cross-linked derivatives have been synthesized and characterized, their interaction with the enzyme under study (lysozyme) permitting to consider their use as supports for the controlled release of biomolecules.

Further studies are in progress for a more advanced characterization of the polysaccharide and for the synthesis and characterization of new derivatives, with the aim of finding new application domains for this new, less expensive biopolymer.

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