Investigation on the Development of Sturdy Bioactive Hydrogel Beads

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Received 3 January 2007; accepted 1 August 2007
DOI 10.1002/app.27445
Published online 20 November 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Biocatalytic hydrogel beads, which retain higher activity, expand, and contract with changes in pH, having biocompatibility, are developed. Composite spherical beads of chitosan having a diameter of 1–2 mm were prepared by ionic gelation using sodium tripolyphosphate (TPP). Above 3% TPP, the activity of the enzyme decreases. The mechanical strength of the chitosan–TPP beads was further improved by the addition of clay or cassava starch granules. The immobilization of protease (fungal, Aspergillus) was done with glutaraldehyde crosslinking. The chitosan–starch hydrogel beads showed significant increase in firmness and stiffness when compared with chitosan–clay beads. The swelling studies show that the particles expand at pH 1.2 and contract at pH 7.4. The activity retention of the immobilized protease was as high as 70% and exhibited a high pH and lower temperature optima than the free enzyme. Chitosan–starch hydrogel beads exhibited degradation peaks at about 90–110°C in TGA analysis. The biocatalyst beads retained 85% of the original catalytic activity even after eight cycles of repeat use. The freeze-dried beads has good storage stability and can be used either as artificial bioreactor systems in detergent or in therapeutic formulations © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 2899–2908, 2008

Key words: chitosan; hydrogel; starch; clay; protease; immobilization; pH sensitive

INTRODUCTION

Research on the functional hydrogels is being actively carried out for a variety of applications such as material for drug delivery system, artificial skin, cosmetic, food and nutrition additives, functional fiber and film, solid state batteries, and water engineering materials.[1] The hydrogels can facilitate maintenance of the desirable enzyme activity, pH, and affinity to the substrate and stability due to the changes in the micro environment or by altering the mobility of the protein. Applications of native enzymes in industrial products and processes are limited due to the instability and rapid loss of biocatalytic activity by autolysis, protein unfolding, and aggregation during the operation and storage. Immobilization of enzymes can offer several advantages over the native enzymes, which include ease of handling, recovery from the reaction medium, and reuse and its utilization in a continuous process. Immobilization of enzymes in biopolymers such as chitosan and starch hydrogels is currently a dynamic area in medicine, food, cosmetics, and in drug delivery applications.[2] These are polymers of glucose and hence nontoxic, available in abundance at a low cost; ease of enzyme accessibility and hence biodegradable, hydrophilic character, and abundance of hydroxyl groups on the surface capable of interaction with proteins. Chitosan is a linear copolymer of D-glucosamine and acetyl-D-glucosamine having β (1 → 4) linkage is an interesting biomaterial due to good biocompatibility,[3] biodegradability,[4] and low toxicity, haemostatic and anti-inflectional activity[5,6] and is used in the immobilization and controlled release of biological compounds. The amino groups of chitosan facilitate the immobilization of enzyme either by adsorption or by chemical reaction[7] and have been shown to be a superior support, compared to polysaccharides such as alginate.[8,9] In addition, chitosan exhibits a significant protein-binding capacity and a high recovery of enzyme activity, allowing the immobilized enzyme to be highly active.[10] Chitosan–alginate complex hydrogel bead using TPP as the crosslinking agent for the controlled release application has been reported in the literature.[11] Kaolinite clays have many useful features for an enzyme immobilization matrix. Pure kaolinite (Al_2O_3-$2SiO_2$·$2H_2O$) is white in color, and its chemical composition is 46.54% SiO_2, 39.50% Al_2O_3, and 13.96% H_2O.[12] The significant intercalation properties of clays, as well as their porosity due to swelling phenomenon in water, allow enzyme immobilization without covalent binding and exhibit a high hydrophilic character[13] and are nontoxic. Proteases are widely used in industrial and biomedical

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Contract grant sponsor: State Government Kerala.

applications, and the immobilization of protease in various biopolymers has been reported.[14] Alkaline protease has been used mainly in the food industry and in detergents. There are various natural nontoxic, biodegradable, and inexpensive supports available for the enzyme immobilization such as cellulose,[15,16] agarose,[17] alginate,[18] and carrageenan[19] with excellent biocompatibility and hydrophilicity. But the lack of mechanical strength of these natural materials has limited their application. We have developed a biocompatible, biocatalytic, sturdy, and chitosan–starch composite hydrogel beads having pH responsiveness. This paper gives the properties of these biocatalytic beads.

MATERIALS AND METHODS

Materials

Chitosan [degree of deacetylation (DD) 84%], glutaraldehyde (25%), and protease (Fungal) were purchased from Sigma-Aldrich (St. Louis, MO). Tripolyphosphate (TPP), trichloroacetic acid, and casein were from S.D. Fine Chemicals (Mumbai, India). Cassava starch (granule size: 5–35 μm, half oval in shape, amylose: 22%, gelatinization temperature: 72°C) and kaolinite clay (English India Clays, Trivandrum, particle size: 2–4 μm) were obtained locally and purified before use. Extran was purchased from Merck. All the other reagents used are of analytical quality.

Preparation of chitosan solution

Chitosan [1.5% (w/v)] was dissolved in 3% acetic acid, filtered through an organdie cloth, and stored at room temperature (28°C ± 3°C).

Preparation of crosslinking solution

The crosslinking solution was four freshly made with 1% (w/v) TPP in distilled water. The pH of the crosslinking solution was 9–10.

Preparation of chitosan–starch–clay hydrogel beads

Starch granules or clay 1% (w/v) was dissolved in chitosan 1.5% (w/v) solution. The air bubble free viscous hydrogel solution was added dropwise, at a constant rate through a syringe into the crosslinking solution of 1% (w/v) TPP at room temperature (28°C ± 3°C). The chitosan–starch–clay–TPP beads were formed instantaneously.[20] The beads were allowed to cure for 8 h in the same solution and washed with distilled water.

Reinforcement of chitosan hydrogel beads by gluteraldehyde treatment

The solidified beads are then crosslinked with 2% (v/v) glutaraldehyde by stirring the beads at 150 rpm at 30°C for 1 h, and the beads were washed thoroughly with water and stored in deionized water.

Texture analysis of hydrogel beads

The mechanical properties of all hydrogel gel bead formulations were examined using a Stable Micro System Texture Analyzer (Model TA-HDi, UK) in texture profile analysis (TPA) mode. Formulations were transferred into the pan. In TPA mode, the analytical probe is compressed into each sample at a definite rate to a depth of 2 mm and then retraced its path. The data acquisition parameters were 10 mm/s prespeed, 1 mm/s test speed, 10 mm/s postspeed, with a distance of 50% strain. The resulting profiles were analyzed using Texture Expert software.

Immobilization of protease on the chitosan–starch–clay hydrogel beads

An amount of crosslinked composite bead was contacted with an appropriate amount of protease enzyme for 2 h at 4°C and agitated in a shaker at 100 rpm. After repeatedly washing with distilled water for three times, the immobilized beads were stored in distilled water at 4°C.

Protease activity assay

Proteolytic activity of native and immobilized protease was assayed at 60°C in Gly-NaOH buffer (50 mM, pH 10.5) using casein as the substrate.[21] One unit of the protease was equivalent to the amount of enzyme required to release one micromole of tyrosine/milliliter/minute. Protein was estimated by Lowry's Method[22] using Bovine Serum Albumin.

The effect of concentration of TPP

The effect of the concentration of TPP on the enzyme activity was studied; the concentration of TPP was changed from 1 to 5% (w/v) maintaining all the other conditions the same.

Determination of optimum temperature, pH, and kinetic parameters

Optimum temperature, pH, K_m, and V_{max} were determined by changing each of the parameters by keeping all the other conditions constant, and the protease activity was assayed. The activity of native

and modified enzyme was studied at a temperature range of 30–80°C at pH 10.5. The activity profile was studied at different pH range of 6.0–10.5 at 60°C using 50 m*M* buffer (pH 6–8, phosphate buffer; 9–10.5, NaOH/glycine buffer). The kinetic studies were done by changing the substrate concentration from 0.25–2.5% (w/v).

Reusability of immobilized protease

To determine the reusability of the immobilized protease, the beads were washed first with water and then with buffer after each usage and then suspended again in a fresh reaction mixture to measure the enzyme activity. The procedure was repeated until the enzyme lost 50% of its original activity (half-life).

Freeze-drying procedure

Freeze drying was accomplished in a lyophilizer. Fresh chitosan beads were freeze dried at −50°C for 6–8 h freezing at or below −50°C was found to minimize damage.[23]

Swelling properties

The swelling properties of the chitosan–TPP composite beads were determined in stimulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) at room temperature (28°C ± 2°C). The percentage of swelling of the beads was calculated from the formula.

$$Q_s = \frac{W_s - W_d}{W_d}$$

where Q_s is the swelling ratio, W_s the weight of swollen bead, W_d is the weight of dry bead.

FTIR

The chemical structures of the chitosan hydrogel beads with starch and clay were characterized by FTIR. The IR spectra were recorded on a PerkinElmer System 2000 FTIR spectrophotometer (PerkinElmer Cetus Instruments, Norwalk, CT) with KBr pellets.

X-ray diffraction study

X-ray patterns of chitosan, chitosan–starch–clay hydrogel beads were powdered and analyzed by X-ray diffractometer (XPERT, Philips, Eindhoven, The Netherlands) in the angular range of 2–40 (2θ) with nickel-filtered Cu Kα radiation (λ = 0.154 nm) employing X'celerator and monochromator at the diffracted beam side at a voltage of 40 kV and current of 30 mA.

Thermal analyses

Thermogravimetric analyses (TGAs) of hydrogels beads were performed in a simultaneous DTA-TG Apparatus (DTG-60, Shimadzu, Kyoto, Japan). Samples (2–8 mg) were heated at the rate of 20°C/min from ambient temperature to 600°C. Nitrogen was used as the purge gas at low rate of 20 mL/min.

Differential scanning calorimetry analyses

Thermal properties of the hydrogel beads were characterized using a PerkinElmer Pyris DSC 6 (PerkinElmer, Boston, MA; DSC: differential scanning calorimetry). Nitrogen at a rate of 30 mL/min was used as the purge gas. Samples (3–6 mg) were taken in aluminum pans, crimped close using the DSC sample press, and heated up to 200°C, ensuring that the samples did not decompose, and cooled to 30°C in the first run followed by a second run in which they were heated to 200°C.

Scanning electron microscopy

The shapes and surface morphology of the beads were examined using electron microscope (JEOL make, model JSM 5600 LV, Tokyo, Japan). For SEM studies, samples were mounted on metal stubs using double-sided adhesive and sputtered with gold. The micrographs were taken at an accelerating voltage of 15 kV and at various magnifications.

Activity retention of chitosan–starch hydrogel beads in detergent solution

To study the activity retention of chitosan–starch hydrogel beads, a definite amount of standard protease free detergent (pH 10) was introduced into the reaction mixture and incubated for a definite period of time, and then the residual enzyme activity was found out. Measurements were also made at the standard conditions to check the effectiveness of the protease containing detergent when diluted in water.

Statistical analyses

All the experiments were done in triplicate. Microsoft Excel 98 (Microsoft, Redmond, WA) was used for all the statistical analysis. Differences are considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Chitosan is known as an ideal support material for enzyme immobilization because of its many characteristics like resistance to chemical degradation, avoiding the disturbance of metal ions to enzyme, and antibacterial property.[24,25] Chitosan–starch–clay composites have produced sturdy smooth, spherical beads due to the ionic interaction between positively charged chitosan and negatively charged TPP[26] at pH 8.0–9.0. The chitosan-TPP beads did not show any tendency to aggregate.[27] The network structure of the matrix of the beads offers maximum binding sites for the covalent immobilization of the enzyme via glutaraldehyde coupling to form a Schiff's base, which in turn makes the binding strong.[28] The average molecular weight (M_w) by measuring the intrinsic viscosity of the chitosan was found out to be 1351 kDa. The beads have a hydrophobic surface with more $—NH_2$ groups that protects the dispersion of chitosan in the medium. Compared to alkali or ethanol gelation, the TPP-chitosan beads are more rigid because of the ionic interactions between $P_3O_{10}^{5-}$ (TPP) and the NH_3^+ (chitosan) in acidic solution.[26] Moreover, addition of either starch or kaolinite clay imparted more rigidity to the beads by intercalating with the polymer network. This improves the mechanical properties of the chitosan beads. Addition of starch also increases the firmness, toughness, and stiffness of the chitosan hydrogels due to the improved net working of the gels forming additional crosslinks with the same crosslinking agent, when compared with the addition of clay. Texture analysis was carried out on freshly prepared hydrogel beads and given in Table I. The chitosan–starch hydrogel beads showed significant increase in firmness and stiffness when compared with chitosan–clay hydrogel and chitosan beads. There was no marked change in the toughness of both the hydrogels beads.

The chitosan beads, when incorporated with 1% of raw cassava starch granules improved its stability and strength. Kaolinite is a layered aluminosilicate mineral with one tetrahydral sheet linked through one octahydral sheet of alumina octahydrate, whereas starch is a polymer of α-1,4 linked glucose having around 20% straight chain amylose and 80% branched chain amylopectin molecules in the semispherical granule of 10–40 μm size. In the chitosan composite beads, the polyelectrolyte complexation occurs between chitosan and TPP, and then with either starch or kaolinite clay by intercalating into the gel matrix or due to the interpenetration of the polymer chains into the gel matrix and thus protecting the gel beads. Chitosan and starch are biocompatible and biodegradable, and its products are nontoxic; they show physiological inertness and significant affinity for proteins. It is also an ecofriendly biomaterial and safe for humans and is biodegradable.

TABLE I
Texture Analysis of Chitosan with Starch and Clay Hydrogel Beads

Treatments	Firmness	Toughness	Stiffness
Chitosan	48.41	14.08	60.97
Chitosan–starch	64.46	16.58	90.57
Chitosan–clay	36.83	11.60	41.56

TABLE II
Activity Retention of Enzyme in Different Matrices

Immobilization	Activity retention (%)
Chitosan	57.3
Chitosan + starch	69.3
Chitosan + clay	61.4

Protease activity retention

The enzyme immobilization efficiency of the matrices was in the range of 57–70% (Table II). Maximum enzyme immobilization efficiency was found to be with chitosan–starch complex beads followed by chitosan–clay beads than chitosan alone.

Effect of TPP concentration on enzyme activity

The beads with maximum enzyme activity were found to be with 1% TPP, was used in the case of chitosan and chitosan–starch complex, however above 3% TPP, the activity of the enzymes decreases (Fig. 1). But the chitosan–clay beads showed maximum activity retention was with when 3% TPP. This

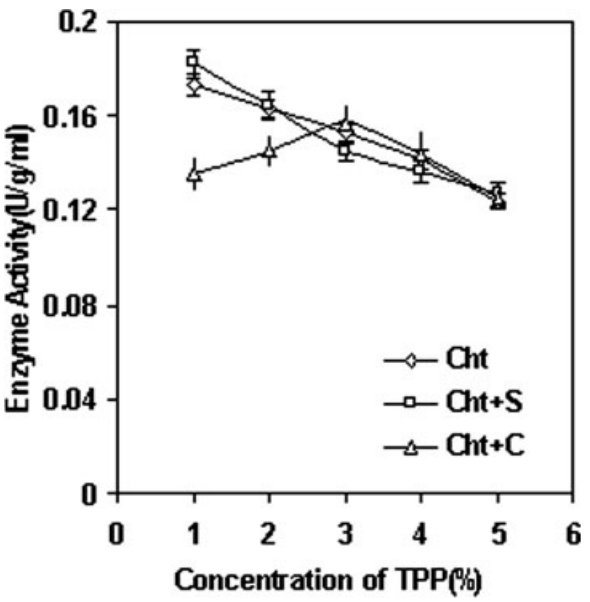

Figure 1 Effect of TPP Concentration on the enzyme activity.

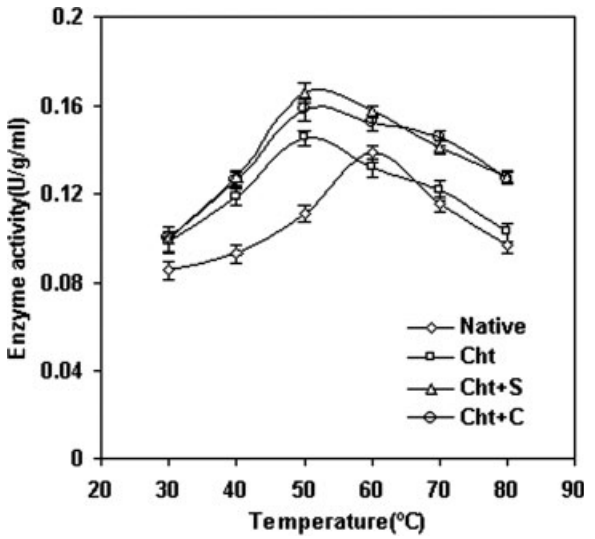

Figure 2 Temperature profile of immobilized protease.

is mainly because of the competition of phosphate groups for the ligand-exchange sites of this layered alumnosilicate mineral.[29] Hence a higher concentration of TPP is needed for the crosslinking of chitosan–clay beads. Crosslinking characteristics of the chitosan–TPP beads were enhanced by the modification of liquid curing mechanism of the beads in TPP solution. The anionic counter ion, TPP, can form either intermolecular or intramolecular linkages; and this is responsible for the successful formation of the beads with lower crystalinity. The glutaraldehyde crosslinking is based on Schiff's base formation and reported to increase the surface hydrophobicity of the beads significantly.[30]

Effect of temperature on enzyme activity

The protease activity of native enzyme increased gradually with temperature, and a maximum activity was obtained at 60°C. But in the case of immobilized enzyme, the maximum activity was obtained at 50°C. This shows a slight modification of the enzyme molecule or the microenvironmental effects due to the immobilization (Fig. 2). The difference in temperature activity profiles between the free and immobilized enzyme is that the latter is more susceptible to temperature-induced conformational changes after immobilization, which is a rare phenomenon in the enzyme immobilization. Immobilization of any enzyme improves the temperature stability due to the rigidity followed by immobilization, which in turn prevents denaturation. There is a 10°C reduction in the optimum temperature, which is mainly due to an increase in the conformational flexibility of the enzyme due to the unique microenvironment prevailing within the immobilized beads.

Effect of pH

The pH is one of the significant parameters capable of shifting enzymatic activities in aqueous solution. Immobilization of enzyme results in the conformational change in the enzyme resulting in a shift in the optimum pH. The effect of pH on the activity of free and immobilized protease preparations was studied at various pH values at 60°C, and the results are shown in Figure 3. The optimum pH of the native enzyme was at 8.0 whereas the immobilized protease was at pH 9.0. This pH shift toward alkaline side upon immobilization is due to the secondary interactions between the coupling agent, the enzyme, and the polymeric gel matrix. The glutaraldehyde coupling of the matrix with the enzyme would have linked all the available amino groups on the surface of the enzyme, and hence the acidic groups on the enzyme surface give a negative charge to the enzyme protein, ultimately shifting the optimum pH to the higher side.

Reusability of the chitosan hydrogel beads

The residual activity of immobilized protease is shown in Figure 4. The immobilized protease beads had better reusability, as it retained 70–85% of the activity even after eight cycles (80 min) of repeated use at 60°C. Chitosan–starch beads showed a better reusability profile and had a half-life of 100 min. The reusability of immobilized protease is essential for cost-effective use of the enzyme either in repeated batch or in continuous processes.

Kinetics of immobilized protease

When the substrate concentration was plotted against the ratio of the substrate concentration to the rate of

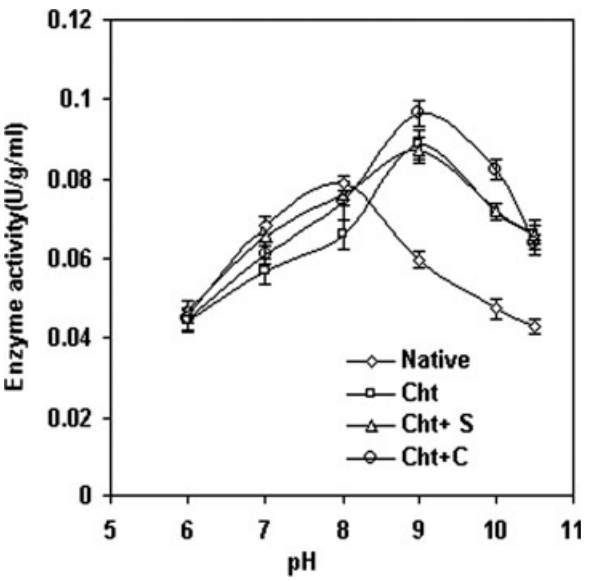

Figure 3 pH profile of immobilized protease.

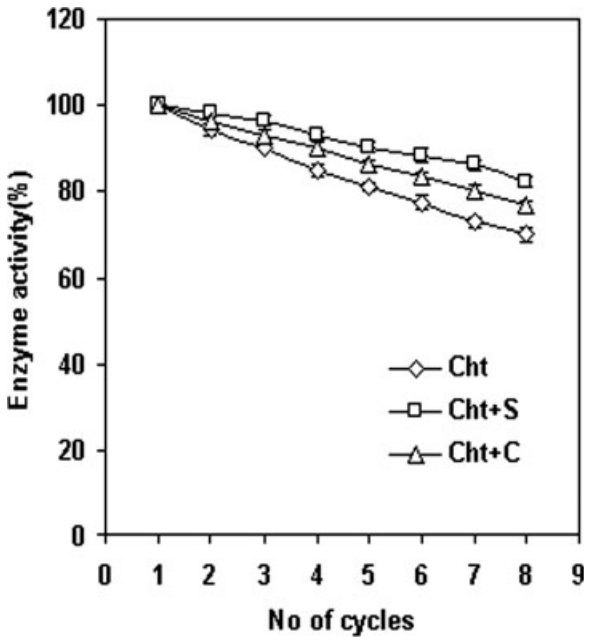

Figure 4 Reusability of chitosan hydrogel complex.

the reaction (Hanes-Woolf plot), the primary plot was obtained and Michaelis-Menton constant (K_m) and velocity maximum (V_{max}) were obtained automatically from the secondary plot values using Sigma plot (7.01 version) 2001 software. Hanes Woolf equation can be represented as $[S]/v = (1/V_{max})[S] + K_m/V_{max}$ where [S] is the substrate concentration, V_{max} is maximal velocity, and K_m the binding of substrate at $1/2V_{max}$ (Michaelis constant). Hanes-Woolf plot avoids both the misleading impression of the experimental error and the uneven distribution of the points by Lineweaver-Burk plot and the angular distortion of the errors of the Eadie-Hofstee plot.[31] The kinetic parameters for the hydrolytic activity of the native and immobilized protease were assayed at substrate concentrations from 0.25 to 2.5%. V_{max} defines the maximum possible velocity when the entire enzyme is saturated with substrate, so, this parameter reflects the intrinsic characteristics of the immobilized enzyme, and could be affected by diffusion constraints. The V_{max} value of the native protease (7.6×10^{-5}) was found to be higher than that of immobilized protease on hydrogel beads. In general, V_{max} values of enzymes demonstrate a decrease upon immobilization. K_m is defined as the substrate concentration that gives a reaction velocity of half the V_{max}. This parameter reflects the effective characteristics of the enzyme and depends upon both partition and diffusion effects. The native and immobilized enzyme showed almost same K_m value (1.1×10^{-5} to 1.3×10^{-5}) showing that there is no change in the affinity of the enzyme toward the substrate (Figs. 5 and 6). However, there is a one-third reduction in the k_{cat} value and is also reflected in the catalytic efficiency of the immobilized enzyme (Table III). This is mainly due to the conformational rigidity and the stearic hindrance in the active site of the enzyme due to the bulky substrate and the microenvironment around the immobilized enzyme.

Swelling studies

The swelling studies show that the particles expand at pH 1.2 and contract at pH 7.4. In hydrogels, the extent of crosslinking heavily depends upon the amount of crosslinking agent used, the pH of the media as well as on the equilibrium swelling. This property is of special interest in biomedical applications such as wound dressings and controlled drug release systems and detergent formulations. Swelling is mainly influenced by ionic interactions between chitosan chains, which depend on the crosslinking density set during the formation of the network. If the pH decreases, the charge density of the crosslinker and the crosslinking density decreases, which in turn leads to swelling. If the pH decrease is too large, the dissociation of ionic linkages and the

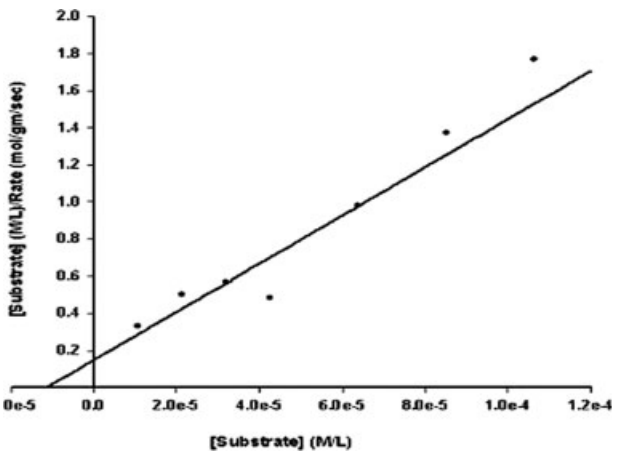

Figure 5 Hanes-woolf plot of native enzyme.

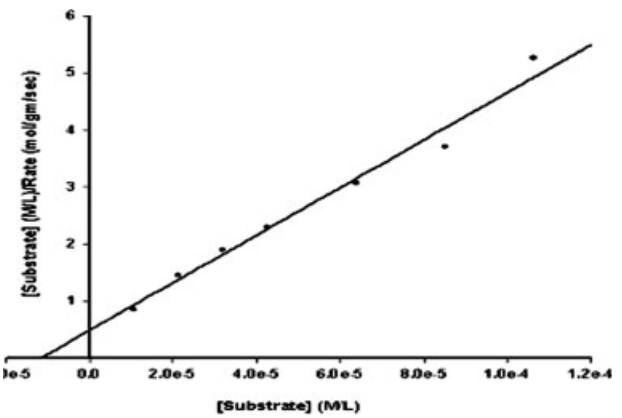

Figure 6 Hanes-woolf plot of chitosan: starch immobilized enzyme.

TABLE III
Kinetic Parameters of Native and Immobilized Protease

Enzyme	K_m (mol/mL)	V_{max} (mol^{-1} s^{-1} g^{-1})	K_{cat} (mol^{-1} s^{-1}/ mole of enzyme)	k_{cat}/K_m
Native	1.1×10^{-5}	7.6×10^{-5}	2.6	2.3×10^{5}
Chitosan	1.3×10^{-5}	2.3×10^{-5}	0.81	5.9×10^{4}
Chitosan + starch	1.1×10^{-5}	2.3×10^{-5}	0.83	7.1×10^{4}
Chitosan + clay	1.2×10^{-5}	2.2×10^{-5}	0.80	6.6×10^{4}

dissolution of the net work can occur, leading to a faster release of the drug. The swelling response of the glutaraldehyde crosslinked chitosan beads in solution at pH 1.2 and pH 7.4 at room temperature (30°C ± 2°C) is shown in Figures 7 and 8.The swelling behavior of the various chitosan beads in acid appears to depend on the ionic-crosslinking density of the chitosan-TPP beads. In the case of chitosan, the amino groups of the polymer allow the establishment of different types of interactions with both nonionic and ionic drugs and also provide pH sensitive systems, which swell at high pH conditions allowing a site-specific release. The mechanism of pH sensitive swelling (when the pH decreases) involves the protonation of the amino groups of the chitosan. This protonation leads to the chain repulsion, diffusion of proton, and counter ions together with water inside the gel and the dissociation of the secondary interactions, allowing the swelling of the hydrogel.

FTIR spectroscopy

FTIR spectroscopy was used to examine the interactions between chitosan starch and clay. The infrared spectra of chitosan, chitosan–starch, and chitosan–clay composite hydrogel beads are presented in Figure 9. The IR spectrum of chitosan hydrogel bead was significantly different from that of chitosan–starch and chitosan–clay hydrogel beds because of the ionization of the primary amino groups. In chitosan beads, the relatively broad band at 3424 cm^{-1} was due to the primary amine.[32] The peaks at 2861 and 2939 cm^{-1} are typical C—H stretch vibrations.[33] The peak at 1719 cm^{-1} suggested the presence of carbonyl group in the hydrogel bead, the one at 1653 cm^{-1} was due to the C=O stretching (amide I), and the peak at 1313 was due to amide III peaks.[34] The peaks at 1423 and 1380 cm^{-1} correspond to the CH_3 symmetrical deformation mode.[35] The broad peak at 1076 cm^{-1} indicates the C—O stretching vibration in chitosan.[36] When two or more substances are mixed, the chemical interactions are reflected by changes in the characteristic spectral peaks. Addition of starch and clay to the chitosan shifted the primary amine group. The peak at 3625 cm^{-1} in chitosan–clay hydrogel bead indicates the O—H stretching. Addition of starch and clay to the chitosan hydrogel beads shifted the peak between 2862 and 2939 cm^{-1}.

XRD studies of chitosan and its composites

Figure 10 shows the crystalline nature of the chitosan, chitosan–starch, and chitosan–clay beads. From

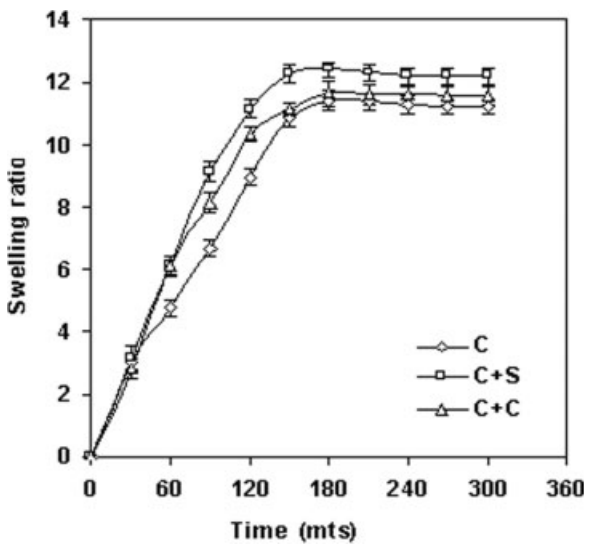

Figure 7 Swelling ratio of crosslinked chitosan/starch/clay beads at pH 1.2.

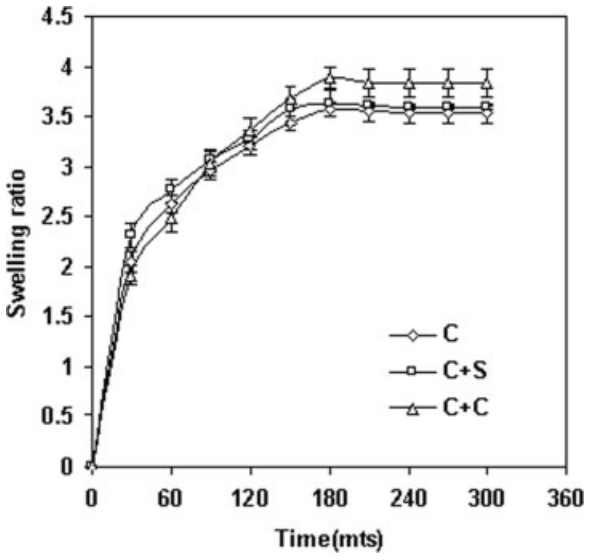

Figure 8 Swelling ratio of crosslinked chitosan/starch/clay beads at pH 7.4.

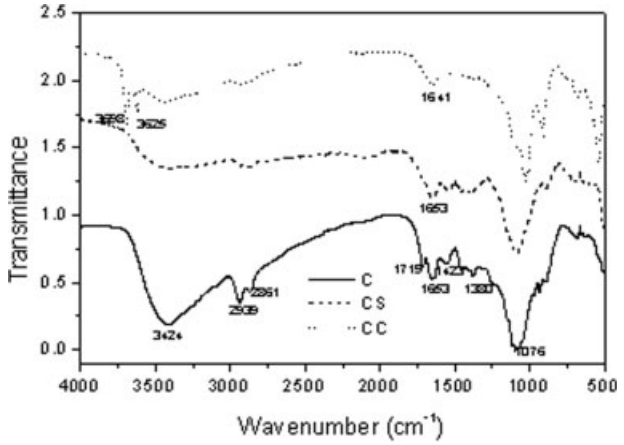

Figure 9 FTIR spectra of chitosan, chitosan–starch, chitosan–clay hydrogel beads.

the figure, it is clear that the chitosan is in a hydrated crystalline state. It shows two diffraction peaks at $2\theta = 15.12^\circ$ and 22.37°. The intensity of the hydrated crystal peak at 15.12° was higher than at 22.37°. In the case of chitosan–clay complex, sharp peak is at $2\theta = 12.35^\circ$ and 24.8°. This sharp peak shows that chitosan–clay beads are in a larger crystalline state than chitosan alone due to the intercalation of the chitosan in the lattices of the clay particles. X-ray diffraction pattern of chitosan–starch bead shows that they are in smaller, less compacted crystals due to the interpenetration of partially amorphous α-linked anhydroglucose units of amylose and the branched amylopectin molecules into the more compact β-linked structure of the chitosan, resulting in a broader peak at the same 2θ of 22°. The crystallinity is maintained on lyophilization of the beads, which retained the crystalline nature of the chitosan in the composite. In the preliminary studies on the various drying methods with protease loaded chitosan–starch–clay hydrogel beads, freeze drying proved to be better than air drying, oven drying, and vacuum drying, in terms of retention of enzyme activity as well as the size and shape of the beads.[37]

Thermal studies of hydrogel beads

Thermoanalytical techniques such as thermogravimetry (TGA) and differential scanning calorimetry (DSC) monitor the physical and chemical changes in both natural and synthetic polymers.[38] These methods yield curves that are unique for a particular composition of matter and changes in chemical structure and composition. The molecular architecture brings about distinct and reproducible variations in the thermogram. Furthermore, some of these changes involve the loss of material in the volatilization, which can be quantitatively measured by TGA. The thermogravimetric curves of the chitosan, chitosan–starch, and chitosan–clay hydrogel beads are shown in Figure 11. From the figure, it can be concluded the chitosan hydrogel beads had a lower thermal degradation temperature than the original chitosan flakes. According to the derivative of TGA, the degradation temperature for 10% weight loss is found to be 75, 98, 71, and 107°C for the samples a, b, c, and d, respectively. Pure chitosan and chitosan starch hydrogel beads exhibited degradation peaks at about 90–110°C. The decomposition temperature of chitosan is molecular weight dependent and low

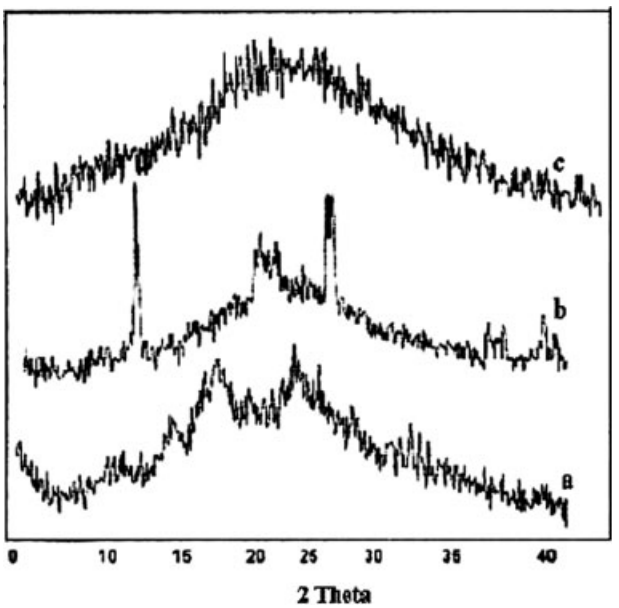

Figure 10 XRD profile of (a) chitosan (b) chitosan–clay and (c) chitosan–starch bead powders.

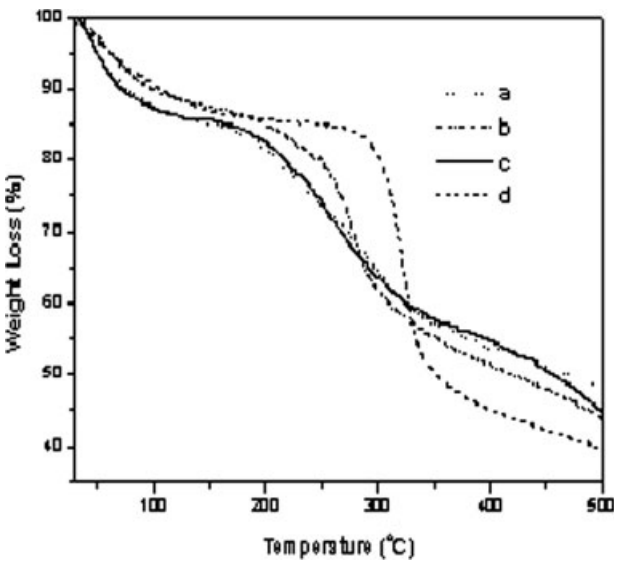

Figure 11 TGA spectra of (a) chitosan hydrogel bead (b) chitosan–starch hydrogel bead, (c) chitosan–clay hydrogel bead, and (d) chitosan powder.

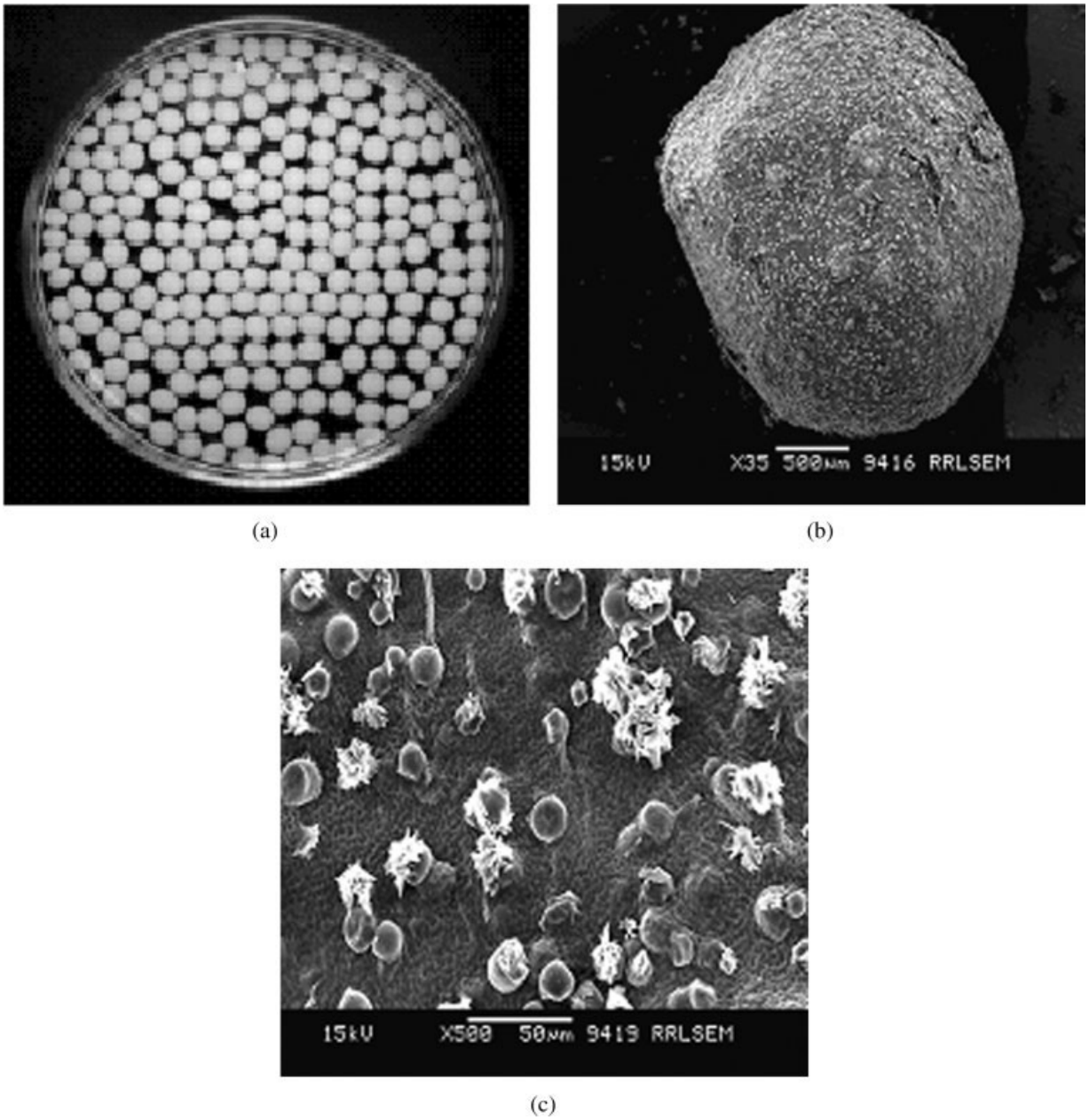

Figure 12 SEM micrographs of (a) Wet beads, size 1–mm (b) freeze dried and (c) surface of the chitosan: starch bead.

molecular weight chitosan degraded at a lower temperature.[39] The DSC melting thermograms did not give any significant data and showed an endothermic peak around 80°C in the first run, which corresponds to the evaporation of water in the samples. Chitosan–clay hydrogel bead has less water of hydration.[40] This behavior is frequently detected in many polysaccharides, such as cellulose and chitin derivatives.[41]

SEM studies

Figure 12 shows the smooth spherical chitosan–starch wet beads and the SEM of (a) freeze dried and (b) surface of the same beads at a higher magnification. The surface morphology of chitosan: a starch composite bead reveals that the enzyme molecules are attached to the globular microstructures of intact starch granules.

Activity retention of hydrogel beads in detergent solution

Detergent enzyme should be stable at high pH and temperature, withstand oxidizing and chelating agents, functional at low enzyme level and have a wide specificity. The wet and dry beads showed good activity retention and retained 50% of the original activity (half-life) after 6 h (Fig. 13). The reduction in activity may be due to the unfavorable

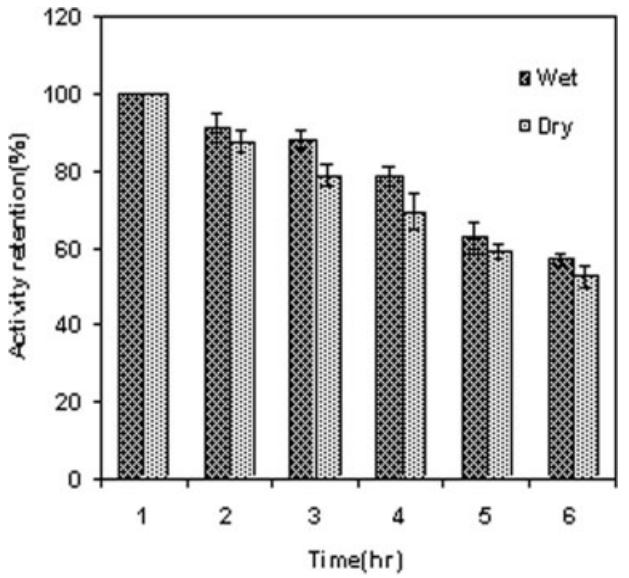

Figure 13 Activity retention of hydrogel beads in detergent solution at different time.

oxidizing and chelating agents and not due to the alkaline pH of the detergent.

CONCLUSIONS

We have developed a biocompatible, biocatalytic, sturdy, chitosan–starch composite hydrogel beads having pH responsiveness. A stable, mechanically stronger hydrogel matrix was prepared by the ionotropic gelation by using a nontoxic biopolymer. Immobilization of protease in the composite chitosan–starch–clay beads has been successfully carried out. The result showed that the activity retention of the immobilized protease was as high as 70%. The pH, thermal, and reuse stabilities of the immobilized enzyme were found to be altered. The chitosan composite beads have good pH stability greater mechanical strength and are active at lower temperature, which is of great interest for use in biochemical reactors, detergent formulation applications, and in the controlled release of drugs. The stability of the beads in the detergent solution was also carried out.

The authors are grateful to the Director, NIST (CSIR), Trivandrum, for providing the necessary facilities for the work, Dr. Peter Koshy, Scientist NIST, for the SEM pictures, Dr. Guruswami, Scientist NIST, for XRD studies, and Drs. S. N. Moorthy and M. S. Sajeev, Scientist CTCRI, Trivandrum, for the texture studies.

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