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Entrapment of enzymes into cellulose–biopolymer composite hydrogel beads using biocompatible ionic liquid

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ABSTRACT

For the first time, lipase from *Candida rugosa* was successfully entrapped into various cellulose–biopolymer composite hydrogels by using a biocompatible ionic liquid, 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]). Lipase-entrapped cellulose and cellulose–biopolymer composite hydrogel beads were simply prepared by co-dissolution of biopolymers in [Emim][Ac] and dispersion of lipase in biopolymer solution followed by formation of biopolymer hydrogel using distilled water. Immobilization yields (specific activity ratio of entrapped lipase to free lipase) of cellulose, cellulose–carrageenan, cellulose–chitosan, cellulose–agarose, and cellulose–agar bead were 35.0, 9.6, 39.7, 41.4, and 52.6%, respectively. Cellulose–biopolymer composite hydrogels proved to be good supports for entrapment of enzymes and have many potential applications, including drug delivery, biosensors, biofuel cells, and tissue engineering due to their inherent excellent biocompatibility and biodegradability.

1. Introduction

Gels are defined as three-dimensional polymer networks swollen by large amounts of solvent. Hydrogels are usually structures formed from natural or synthetic polymers, and contain large amounts of trapped water [1]. Recently, biopolymer-based hydrogels have received considerable attention for applications in biomedical fields, including tissue engineering, drug delivery systems, contact lenses, and biosensors, because of their inherent biocompatibility and biodegradability [2]. Various hydrogels from biopolymers have been fabricated by using hyaluronate, alginate, agarose, starch, gelatin, cellulose, chitosan, and their derivatives.

Cellulose is the most abundant renewable biopolymer. It has excellent thermal and mechanical properties and biocompatibility, and for economic and scientific reasons, is a promising material for biochemical engineering [3,4]. Cellulose hydrogel can be prepared from a cellulose solution through physical cross-linking, because cellulose has abundant hydroxyl groups which can form hydrogen bonds. However, the development of cellulose hydrogel has been hampered by the difficulty of dissolving cellulose, because cellulose is highly crystalline. Recently, ionic liquids (ILs) have been developed to dissolve cellulose, providing great opportunities for the preparation of cellulose hydrogels. ILs are organic salts that usually melt at temperatures <100 °C. Interest in ILs stems from their potential applications as 'green solvents'. ILs are good solvents for polar organic, nonpolar organic, inorganic, and polymeric compounds [5]. Cellulose hydrogels have been prepared by regenerating a cellulose solution in 1-allyl-3-methylimidazolium chloride ([Amim][Cl]) using deionized water as a coagulant [6]. Additionally, biopolymers such as chitin, chitosan, silk, and DNA can be fabricated from ILs to produce films, membranes, fibers, spheres, and molded shapes [4]. Therefore, various biopolymer composite hydrogels can also be prepared by co-dissolution of two or more biopolymers into ILs. Recently, Sun et al. [7] prepared cellulose–chitosan composite beads using 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) for heavy metal ion adsorption. Blending of different biopolymers is an extremely attractive inexpensive and advantageous method to obtain new structural materials. Cellulose-based composite hydrogels blended with various biopolymers will create novel materials for special applications [1,8].

Enzymes have been recognized as efficient and environmentally friendly catalysts because of their high specificity and catalytic activity under mild conditions. However, the industrial applications of enzymes have been limited due to their low stability, and difficult recovery for subsequent use. Enzyme immobilization is the most commonly used strategy to overcome these drawbacks [9]. Entrapment, one of the immobilization techniques, can be defined as physical restriction of an enzyme within a confined polymer network, and unlike support binding, requires the synthesis

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of a polymeric network in the presence of enzymes [9]. Various polysaccharide hydrogels such as alginate, chitosan, agarose, and carrageenan have been employed for the entrapment of a number of enzymes such as lipases, lactases, invertase, endo-β-glucanase, and peroxidase [10–14]. However, at this time, there are few reports on the entrapment of enzymes into non-derivatized cellulose. Even though microbial cells, not isolated enzymes, were entrapped within a cellulose fiber and beads with a mixture of *N*-ethylpyridinium chloride and dimethylformamide [15,16], the resulting fiber was generally brittle due to insufficient gelation [17]. Recently, Turner et al. [18] attempted to use ionic liquid [Bmim][Cl] to entrap laccase into a cellulose membrane. The enzyme was entrapped into cellulose but showed low residual activity because of IL-induced denaturation. It was known that ILs capable of dissolving cellulose also have a denaturing effect on enzymes. It is expected that the activity of the entrapped enzyme could be enhanced by using ILs, which cannot only dissolve cellulose but also do little harm to enzymes.

In this study, for the enzyme entrapment we used 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]), which is known to be one of the best solvents for lignocellulosic materials among the ILs [19,20]. In addition, [Emim][Ac] is one of the most promising candidates for industrial applications due to its low viscosity, low melting point, non-toxicity, and biodegradability [19,21]. Lipase from *Candida rugosa*, which has more industrial applications than any other enzymes: pharmaceutical, cosmetics, food, perfumery, and bioremediation [22], was entrapped into cellulose hydrogel beads with high residual activity. Moreover, the lipase was successfully immobilized in various cellulose composite hydrogel beads formed with agarose, chitosan, carrageenan, and agar as a counter biopolymer. To the best of our knowledge, this is the first report concerning the successful entrapment of enzyme into non-derivatized cellulose–biopolymer composite hydrogels.

2. Materials and methods

2.1. Materials

Cellulose (microcrystalline), chitosan (high molecular weight, deacetylation degree of 75%), carrageenan (Type I, predominantly κ and lesser amounts of λ carrageenan), 1-ethyl-3-methylimidazolim acetate ([Emim][Ac]), *p*-nitrophenyl butyrate, *p*-nitrophenol, isopropanol, and lipase from *C. rugosa* were purchased from Sigma–Aldrich (St. Louis, MO, USA). Agarose was purchased from Biopure (Ontario, Canada). Agar (gel strength 500–1000 g/cm^2) and sodium alginate were purchased from Samchun Pure Chemical (Seoul, Korea). All other chemicals used in this study were of analytical grade and used without further purification.

2.2. Preparation of cellulose–biopolymer composite hydrogel beads containing lipase

To prepare cellulose hydrogel beads, 5, 6, and 7% of cellulose were dissolved in 5 mL of [Emim][Ac] under stirring at 80 °C for 3 h. Transparent cellulose solutions were dried under vacuum at 60 °C to remove air bubbles. One milliliter of each cellulose solution was mixed with 100 mg of the lipase powder at room temperature. The resultant mixture was then added drop-wise into 1 L of distilled water with vigorous stirring at a rate of 50 μL/min, and accomplished using a 5 mL plastic syringe with a 26-gauge needle and a syringe pump (LSP01-2A, Longer Pump, China). The hydrogel beads were cured in distilled water for 1 h. Washing was conducted 3 times, and the absence of [Emim][Ac] was confirmed by measuring the optical density of the washing solution at 211 nm.

To prepare cellulose–biopolymer composite hydrogel beads, 55 mg of chitosan, carrageenan, agar, and agarose were dissolved in 5 mL of [Emim][Ac] with vigorous stirring at 80 °C for 12 h. After the clear biopolymer solutions were obtained, 275 mg of cellulose was mixed with each biopolymer solution and the mixtures were then stirred at 80 °C for an additional 2 h. Yellowish and transparent cellulose–biopolymer solutions were dried under vacuum at 60 °C to remove air bubbles. Each cellulose–biopolymer solution (1 mL) was mixed with 100 mg of the lipase powder at room temperature. The resultant mixture was then used to make cellulose–biopolymer hydrogel beads by the same procedure used for preparation of cellulose hydrogel beads.

2.3. Determination of entrapped protein content and lipase activity

The amount of protein entrapped in the beads was measured with the Micro BCA^{TM} Protein Assay Kit (Thermo Scientific, USA) using standard protocol. Each cellulose–biopolymer composite bead was also prepared without the lipase to be used as a background for BCA assay. Hydrolytic activity of entrapped lipase was determined by spectrophotometric assay [23]. A hydrogel bead containing lipase was placed in a 50 mL Falcon tube together with 10.5 mL of 0.1 M phosphate buffer (pH 7.0). The reaction was started by adding 0.5 mL of substrate solution prepared by dissolving 10 mM *p*-nitrophenyl butyrate in isopropanol and carried out at 25 °C in a water bath with shaking at 150 rpm. Periodically, 300 μL of aliquots were removed, diluted with 300 μL of acetonitrile, and then centrifuged to obtain supernatant. The activity was expressed as the initial rate and determined by measuring the increase in absorbance at 405 nm by the *p*-nitrophenol produced during the lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate. The initial rate measurements were carried out in triplicate. After reaction for 1 h, hydrogel beads were washed with phosphate buffer (0.1 M, pH 7.0) and their residual activities were determined by subsequent reaction.

2.4. Bead characterization

The size of the composite hydrogel beads was measured using a digital caliper (Fuso, Japan). The diameter of each bead was measured at 3 different angles and averaged. In this way, 10 beads were used to provide an average bead size. The dry weight of the composite beads was measured after drying at 60 °C for 12 h. The surface of the freeze-dried beads was studied using scanning electron microscopy (JSM 6308, JEOL, Japan). All samples were sputter-coated with gold prior to observation.

3. Results and discussion

3.1. Entrapment of lipase in cellulose hydrogel beads

Preparation of enzyme-entrapped cellulose hydrogel has been limited by difficulty in selecting a suitable solvent to dissolve cellulose without inactivating enzyme. Although [Bmim][Cl] was used to entrap laccase, the residual activity was only ~18%, due to enzyme denaturing conditions by [Bmim][Cl] containing a high concentration of [Cl$^-$] [18,24]. In this study, [Emim][Ac], which was used as an enzyme-friendly co-solvent for resolution of amino acids [25], was first employed to dissolve cellulose and entrap enzyme. For the preparation of cellulose hydrogel beads, extrusion of over 8% cellulose solution from a syringe needed too much time and the formed cellulose beads showed irregular spherical shapes, while hydrogel beads formed from less 4% cellulose solution were easily breakable. Finally, 5% cellulose solution was mixed with the lipase and then coagulated by distilled water. The lipase was successfully

Table 1
Influence of biopolymer composites on the hydrolytic activity of entrapped lipase.

Biopolymer concentration in [Emim][Ac]	Activity ($\times 10^{-3}$ μmol/min/bead)	Protein content (μg protein/bead)	Specific activity (μmol/min/mg protein)	Immobilization yield (%) [a]	Wet bead size (mm)	Dried bead weight (mg) [b]	Loaded protein (mg protein/mg)	Residual activity after reuse (%)
5% cellulose	34.7 ± 1.5	20.1 ± 0.8	1.73 ± 0.14	24.0	2.0 ± 0.1	0.50	0.040	85.6 ± 1.8
6% cellulose	52.7 ± 3.4	20.9 ± 2.0	2.52 ± 0.05	35.0	2.0 ± 0.0	0.52	0.040	83.6 ± 6.4
7% cellulose	72.5 ± 5.2	27.9 ± 0.7	2.60 ± 0.23	36.1	2.1 ± 0.1	0.64	0.044	81.8 ± 0.5
5% cellulose, 1% carrageenan	20.2 ± 1.7	29.3 ± 1.3	0.69 ± 0.10	9.6	2.2 ± 0.0	0.53	0.055	90.0 ± 1.9
5% cellulose, 1% chitosan	46.6 ± 2.8	16.3 ± 0.2	2.86 ± 0.15	39.7	2.1 ± 0.0	0.64	0.025	74.4 ± 4.9
5% cellulose, 1% agarose	78.0 ± 3.6	26.2 ± 1.3	2.98 ± 0.20	41.4	2.1 ± 0.1	0.52	0.050	95.9 ± 4.0
5% cellulose, 1% agar	103.7 ± 6.7	27.4 ± 2.9	3.79 ± 0.25	52.6	2.4 ± 0.0	0.54	0.051	88.4 ± 6.4

[a] Immobilization yield (%) = (specific activity of entrapped lipase/specific activity of free lipase) × 100.
[b] Average weight of a bead calculated from 20 beads dried at 60 °C for 12 h.

entrapped into cellulose hydrogel beads and the formed spherical beads showed sufficient rigidness. The entrapped lipase showed activity of 34.7×10^{-3} μmol/min per a bead and the entrapped protein content was 20.1 μg per a bead (Table 1). The specific activity of free lipase was 7.20 μmol/min/mg protein when *p*-nitrophenyl butyrate was used as a substrate. Therefore, immobilization yield, which is defined as a ratio of specific activity of entrapped lipase to that of free lipase, for the cellulose hydrogel beads was 24%. On the basis of dry weight, maximum loaded protein per a bead was 4 wt%. By increasing cellulose concentration from 5% to 7%,

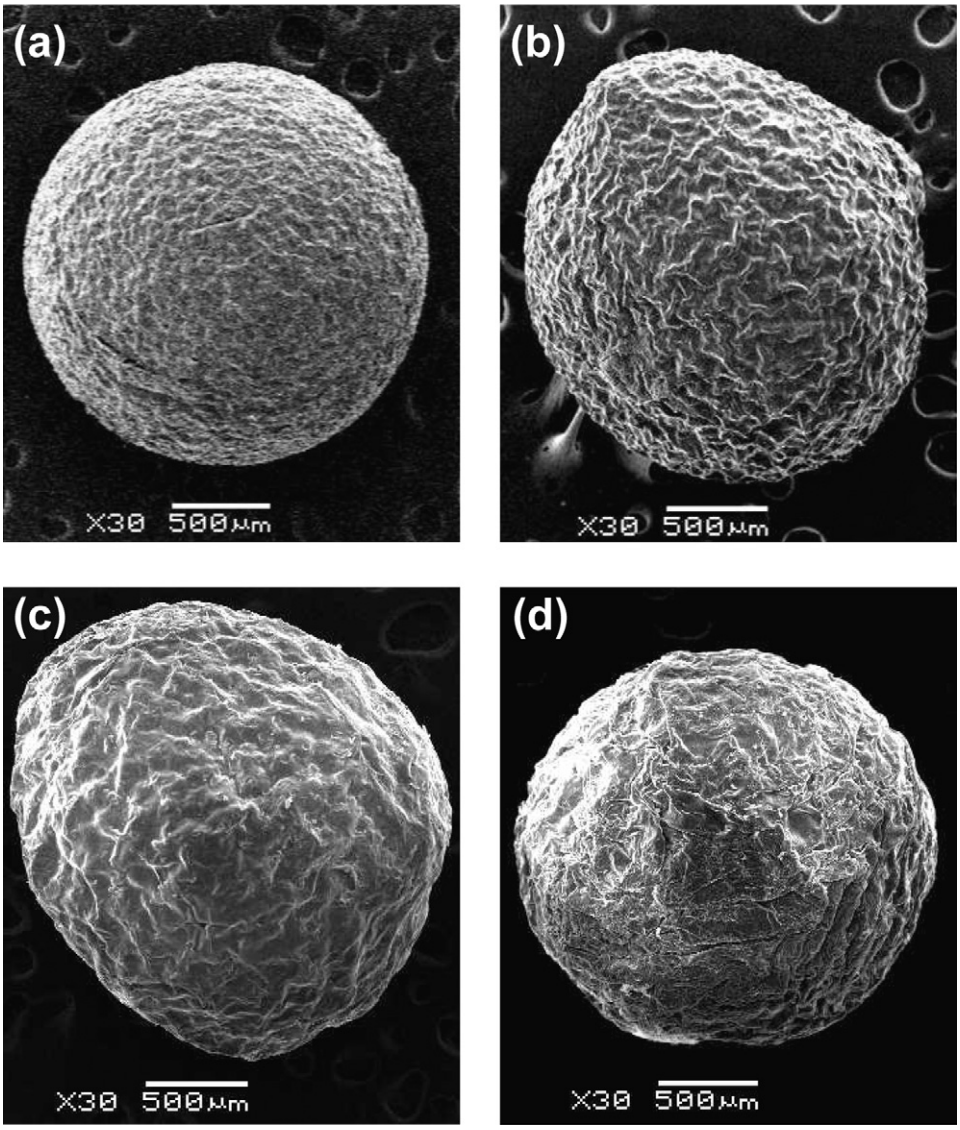

Fig. 1. Scanning electron micrograph of freeze-dried cellulose–biopolymer beads. (a) Cellulose, (b) cellulose–chitosan, (c) cellulose–agar, and (d) cellulose–agarose.

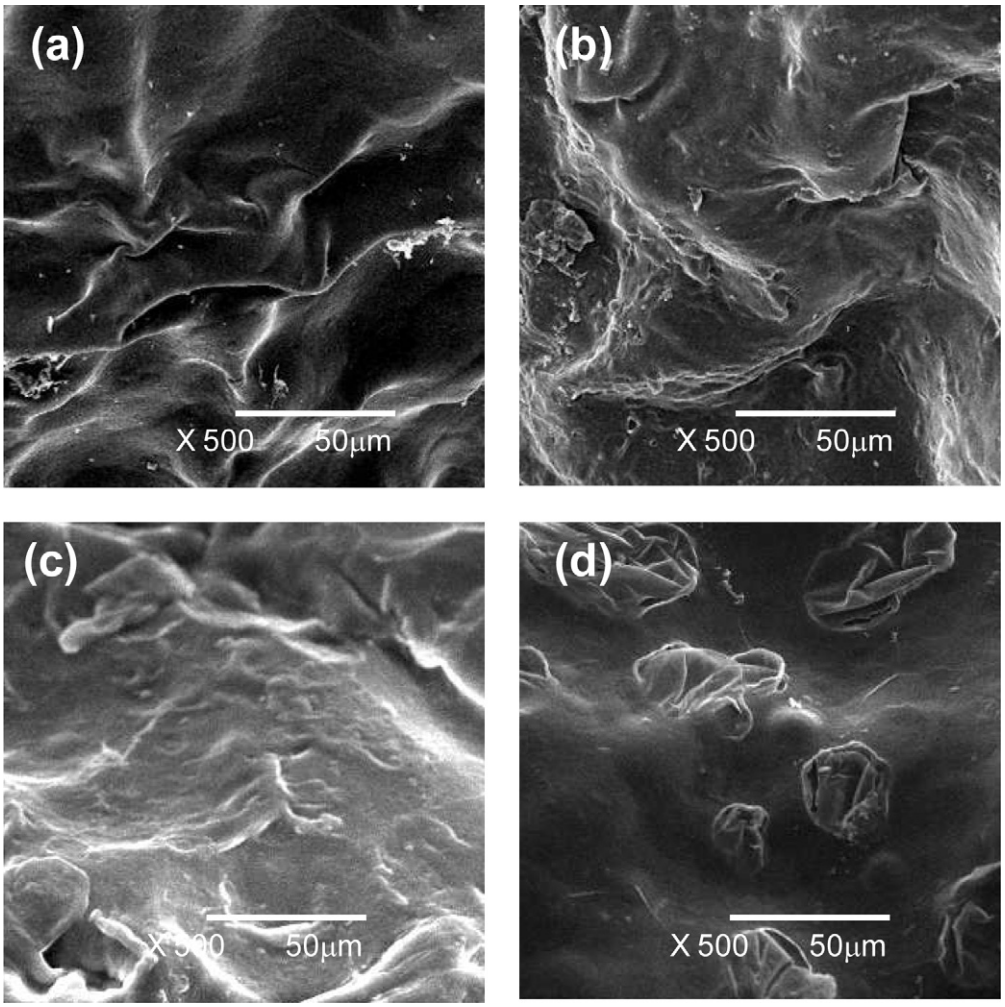

Fig. 2. Enlarged scanning electron micrograph of freeze-dried cellulose–biopolymer beads. (a) Cellulose, (b) cellulose–chitosan, (c) cellulose–agar, and (d) cellulose–agarose.

activity, loaded protein content, specific activity, immobilization yield, size, and weight of hydrogel bead containing lipase were increased. Increased bead size and weight can be simply explained by the increased viscosity of the cellulose solution. Enhanced loaded content of protein with increasing cellulose concentration was similarly shown in the entrapment of lipase into alginate beads in our previous results [11]. However, the increased immobilization yield with increasing cellulose concentration should be further studied, because enzymes entrapped with higher concentration of biopolymer generally showed lower specific activity due to the conformational change of enzyme and/or limitation of substrate transfer [10,11]. Possible reasons for enhanced immobilization yield might be the protection of lipase from unfavorable conditions of [Emim][Ac] by high concentrations of cellulose. The introduction of [Emim][Ac] enabled the lipase to be entrapped in cellulose hydrogel beads with high immobilized yields.

3.2. Entrapment of the lipase in cellulose–biopolymer composite hydrogel beads

Development of composites containing two or more biopolymers is a simple and attractive method to obtain new functional materials. Several cellulose composites were prepared by blending with biopolymers such as starch, alginate, and chitosan. However, cellulose–biopolymer composites were usually prepared with chemically functionalized cellulose or an interpenetrating method, because mutual dissolution of two or more biopolymers containing cellulose in the same solvents is very difficult and gelation conditions for different biopolymers are not the same. To prepare cellulose–biopolymer composites, ILs can be used as a good solvent due to their high dissolution power for biopolymers. Recently, we prepared cellulose–chitosan fiber by electrospinning using IL as a solvent [26]. Both cellulose and chitosan were successfully dissolved in [Emim][Ac] and simply coagulated with ethanol. In this study, to prepare cellulose–biopolymer composite hydrogels, the solubilities of various biopolymers such as agar, agarose, chitosan, carrageenan, and alginate in [Emim][Ac] were first measured. The solubilities of agar, agarose, chitosan, and carrageenan in [Emim][Ac] were ~3, 5, 4, and 2 wt%, respectively. Solubility of sodium alginate was too low to be measured. Both cellulose (5%) and each biopolymer (1%) were dissolved in [Emim][Ac] and cellulose–biopolymer hydrogels were successfully prepared by using the same procedure to make cellulose hydrogel. The lipase was also entrapped into various cellulose–biopolymer composite hydrogels. Table 1 shows the effects of cellulose–biopolymer composite hydrogels on the entrapped lipase. The cellulose–agar hydrogel showed the highest activity, specific activity, and immobilization yield of the tested cellulose beads and cellulose–biopolymer composites. Betigeri and Neau [10] used chitosan, alginate, and agarose hydrogel to entrap lipase. They reported that agarose beads proved to be susceptible to swelling and disintegration. Pure agar may not be suitable for enzyme entrapment, because agar is a mixture of agarose and agaropectin. However, cellulose–agar hydrogel prepared in

this study showed sufficient hardness and high specific activity. While the cellulose–chitosan beads exhibited the lowest loaded protein, the cellulose–carrageenan beads showed the highest protein loading, but the specific activity of the entrapped lipase was the lowest. The possible reason for poor immobilization yield in the cellulose–carrageenan beads might be a polymeric interaction with the lipase, either physical or ionic in nature. The possibility of interaction of the carrageenan specifically with the active lipase site cannot be excluded. As a result, the immobilization yield of cellulose–biopolymer composite was increased in the order of carrageenan < chitosan < agarose < agar. Cellulose–biopolymer composite beads, except for cellulose–carrageenan, showed higher immobilization yields than cellulose beads. These results indicate that cellulose–biopolymer composite hydrogels can be used as more efficient supports for enzyme entrapment than cellulose hydrogel. When the cellulose–biopolymer composite beads were compared in terms of the bead size and weight, the swelling degrees of agar and carrageenan was greater than those of cellulose and chitosan. This may be caused by higher water solubilites of agar and carrageenan.

The dependence of bead morphology on the biopolymer was also examined. The surfaces of cellulose–biopolymer beads were observed using a scanning electron microscope and the SEM images are shown in Figs. 1 and 2. Freeze-dried cellulose beads showed a smooth surface and its shrinkage was minimum among the tested cellulose–biopolymer beads. The cellulose–chitosan beads showed a tougher surface than other cellulose–biopolymer beads. The surface of cellulose–agarose bead was heterogeneous. However, all cellulose–biopolymer beads did not shrink to a great extent during a freeze-drying process. These results mean that the swelling degree or surface characteristics of a biopolymer hydrogel can be controlled by blending two or more different biopolymers using ILs.

The residual activities after reuse of lipase-entrapped cellulose–biopolymer beads were measured (Table 1). It showed that the release of entrapped lipase from cellulose–biopolymer beads was not severe, and the operational stability of entrapped lipase may be controlled by changing the kind and content of biopolymer. Cellulose–agarose bead showed the highest residual activity, while the residual activity of cellulose–chitosan bead was lowest among tested cellulose–biopolymer beads.

4. Conclusions

We have achieved entrapment of lipase into cellulose hydrogel beads with high immobilization yields by using [Emim][Ac] as a dissolving solvent of cellulose. The lipase could be also entrapped into various cellulose–biopolymer composite hydrogels with higher immobilization yields than cellulose beads. Our procedure for enzyme entrapment in biopolymer hydrogels was simple and mild: dissolution of biopolymers in [Emim][Ac], dispersion of enzyme in the biopolymer solution, followed by reconstitution of biopolymers with water for hydrogel formation. Considering that cellulose–biopolymer hydrogels have many favorable properties such as hydrophilicity, biodegradability, biocompatibility, transparency, low cost, and non-toxicity, the present study has wide applications for controllable drug delivery, protein therapeutics, biosensors, biofuel cells, and tissue engineering.

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References

[1] C. Chang, L. Zhang, Carbohydr. Polym. 84 (2011) 40–53.
[2] S.V. Vlierberghe, P. Dubruel, E. Schacht, Biomacromolecules 12 (2011) 1387–1408.
[3] P. Gemeiner, V. Stefuca, V. Bales, Enzyme Microb. Technol. 15 (1993) 551–566.
[4] S.H. Lee, M. Miyauchi, J.S. Dordick, R.J. Linhardt, in: S.V. Malhotra (Ed.), Ionic Liquid Applications: Pharmaceuticals, Therapeutics, and Biotechnology, ACS Symp. Ser., Oxford University Press, 2010, pp. 115–134.
[5] S.H. Lee, T.V. Doherty, R.J. Linhardt, J.S. Dordick, Biotechnol. Bioeng. 102 (2009) 1368–1376.
[6] L. Li, Z.B. Lin, Y. Xiao, Z.Z. Wan, S.X. Cui, Chin. Sci. Bull. 54 (2009) 1622–1625.
[7] X. Sun, B. Peng, Y. Ji, J. Chen, D. Li, AIChE J. 55 (2009) 2062–2069.
[8] I. Šimkovic, Carbohydr. Polym. 74 (2008) 759–762.
[9] R.A. Sheldon, Adv. Synth. Catal. 349 (2007) 1289–1307.
[10] S.S. Betigeri, S.H. Neau, Biomaterials 23 (2002) 3627–3636.
[11] K. Won, S. Kim, K.J. Kim, H.W. Park, S.J. Moon, Process Biochem. 40 (2005) 2149–2154.
[12] M. Matto, Q. Husain, J. Mol. Catal. B: Enzym. 57 (2009) 164–170.
[13] K.R. Jegannathan, E.S. Chan, P. Ravindra, J. Mol. Catal. B: Enzym. 58 (2009) 78–83.
[14] B. Cheirsilp, P. Jeamjounkhaw, A. H-Kittikun, J. Mol. Catal. B: Enzym. 59 (2009) 206–211.
[15] Y.Y. Linko, R. Viskari, L. Pohjola, P. Linko, J. Solid Phase Biochem. 2 (1977) 203–212.
[16] Y.Y. Linko, K. Poutanen, L. Weckstrom, P. Linko, Enzyme Microb. Technol. 1 (1979) 26–30.
[17] Y. Kurokawa, Polym. Gels Networks 4 (1996) 153–163.
[18] M.B. Turner, S.K. Spear, J.D. Holbrey, R.D. Rogers, Biomacromolecules 5 (2004) 1379–1384.
[19] P. Mäki-Arvela, I. Anugwom, P. Virtanen, R. Sjöholm, J.P. Mikkola, Ind. Crop Prod. 32 (2010) 175–201.
[20] N. Sun, M. Rahman, Y. Qin, M.L. Maxim, H. Rodríguez, R.D. Rogers, Green Chem. 11 (2009) 646–655.
[21] N. Sun, H. Rodríguez, M. Rahman, R.D. Rogers, Chem. Commun. 47 (2011) 1405–1421.
[22] S. Benjamin, A. Pandey, Yeast 14 (1998) 1069–1087.
[23] S.H. Lee, T.T.N. Doan, S.H. Ha, W.J. Chang, Y.M. Koo, J. Mol. Catal. B: Enzym. 47 (2007) 129–134.
[24] S.H. Lee, S.H. Ha, S.B. Lee, Y.M. Koo, Biotechnol. Lett. 28 (2006) 1335–1339.
[25] H. Zhao, L. Jackson, Z. Song, O. Olubajo, Tetrahedron Asymm. 17 (2006) 2491–2498.
[26] T.J. Park, Y.J. Jung, S.W. Choi, H. Park, H. Kim, E. Kim, S.H. Lee, J.H. Kim, Macromol. Res. 19 (2011) 213–215.