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Preparation of Cross-Linked Glucoamylase Aggregates Immobilization by Using Dextrin and Xanthan Gum as Protecting Agents

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Abstract: In this paper glucoamylase from *Aspergillus niger* was immobilized by using a modified version of cross-linked enzyme aggregates (CLEA). The co-aggregates were cross-linked with glutaraldehyde; meanwhile dextrin and xanthan gum as protecting agents were added, which provides high affinity with the enzyme molecules. The immobilized glucoamylase was stable over a broad range of pH (3.0–8.0) and temperature (55–75 °C); dependence shows more catalytic activity than a free enzyme. The thermostability, kinetic behavior, and first-order inactivation rate constant (k_i) were investigated. The two types of protector made the immobilized glucoamylase more robust than the free form. Both of the immobilized enzymes have excellent recyclability, retaining over 45% of the relative activity after 24 runs. In addition, immobilized enzymes reduced only 40% of the initial activity after three months by the storability measure, indicating high activity.

Keywords: glucoamylase; cross-linked enzyme aggregates (CLEA); immobilized enzyme; protecting agents; substrate

1. Introduction

The industrial preparation of glucose involves a preliminary starch saccharification to maltodextrin that uses α-amylase, and a second hydrolysis to glucose that uses glucoamylase [1,2]. Glucoamylase (1,4-α-glucanglucohydrolase, EC 3.2.1.3) is an enzyme preparation that decomposes starch into glucose by tearing-off glucose units from the non-reduced end of the polysaccharide chain. As an extra-cellular enzyme, it catalyzes the hydrolysis of α-1,4glucosidic bonds in starch by progressively chopping off single glucose molecules from the ends of amylose chains. Some glucoamylases can also attack the branching α-1,6 bonds of amylopectin, but at a much slower rate than α-1,4 bonds [3]. Immobilized enzyme preparations are useful catalysts for industrial biotransformations [4,5]. Immobilization of enzymes through covalent attachment has also been demonstrated to induce higher resistance to temperature, denaturants, and organic solvents in several cases [6–8]; therefore it has become a hot topic studied by scholars. The carrier bonding of enzymes may produce alterations in their observed activity, specificity, or selectivity [9]. These alterations in enzyme properties are sometimes associated with changes in the enzyme structure. Otherwise, a stabilizing hydrophilic microenvironment can be created by the introduction of hydrophilic macromolecules into the proximity of the enzyme. The stability of an immobilized enzyme is dictated by many factors such as the number of bonds formed between the enzyme and carrier [10]. The development of oxide-coated

glass has increased its durability and half-life, which are important economic advantages, but the cost of the carrier still exceeds the value of the final product [11].

Currently, a large variety of matrixes have been used in immobilized glucoamylase [7,12–14]. The enzymes are linked to an insoluble matrix by chemical bonds, which generally produce very stable derivatives in which enzyme leakage is prevented. The result shows that the stability and recovery times of immobilized enzyme were not ideal. The binding capacity and catalytic ability of the enzyme are reduced when the carrier is present, but the ability to adapt well to the microenvironment was enhanced [15]. In recent years, the advent of self-immobilization [16–18] has intrigued researchers. It has two forms, direct cross-linking and indirect cross-linking. There are disadvantages to the use of direct cross-linking of untreated enzyme molecules such as poor mechanical performance, small granules, and low enzymatic activity By contrast, pre-treatment of enzymes by physical or chemical precipitation or adding protectants makes them more robust for the same cost. Enzymes of cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) [19,20] were precipitated from an aqueous solution by adding a salt or a water-miscible organic solvent or polymer, and then the physical aggregates of enzyme molecules were cross-linked with a bifunctional agent. Higher stability and suitability of immobilized enzymes were achieved by using two types of unconventional methods. CLEA have been applied extensively in many kinds of enzymes [8,21] and represent one of the best potential methods of immobilizing enzymes.

Xanthan gum is a nature polysaccharide and an important industrial biopolymer [22]. It has been used in a wide variety of enzyme modifications for a number of important reasons, such as emulsion stability, temperature stability, and pseudoplastic rheological properties [23]. The storage stability and tolerance of immobilized enzymes were enhanced significantly with xanthan gum in previous studies. The conformation stability of enzymes is necessary for their bioactivity. In the case of CLEA with dextrin co-aggregates, the amino group of the enzyme might react with glutaraldehyde to establish enzyme–enzyme, starch–grain, and enzyme–starch linkages. In this study, dextrin and xanthan gum as protecting agents were added in the process of prepared CLEA of glucoamylase, and we focus on the characteristics of modified CLEA and free glucoamylase. The morphology and tolerance were analyzed by the comparison of immobilized and unmodified enzyme. Furthermore, the thermal stability and the optimum concentration of the protective agent and glutaraldehyde solution were studied systematically in this paper. We also examined immobilized and native glucoamylase with respect to kinetic parameters, recoverability, and performance in different conditions.

2. Results and Discussion

2.1. Glutaraldehyde Concentration

Glutaraldehyde is a reagent mostly used for cross-linking protein molecules [8,24]; however, it induces changes in protein catalytic activities through penetrating the internal structure of the protein and reacting with amino residues. After the aggregates formed, glutaraldehyde in the concentration range of 0.2%–9% (v/v) was added to measure the optimal conditions in Figure 1. It was observed that the maximum activity yield was obtained with 0.5% (v/v) cross-linker concentration and the highest activity of CLEA related to the native enzyme was nearly 76%. As seen in Figure 1, the activity yield of the immobilized enzyme decreased sharply with an increasing degree of glutaraldehyde, while most of the bioactivity was lost at a 9% (v/v) glutaraldehyde concentration, and the robust mechanical strength of CLEA indicated that too much extensive cross-linking occurs and the enzyme loses the minimum flexibility necessary for its activity [25]. CLEA was inflated through preparation in a high-level cross-linker agent solution that destroys active sites of the enzyme in the process of cross-linking [26].

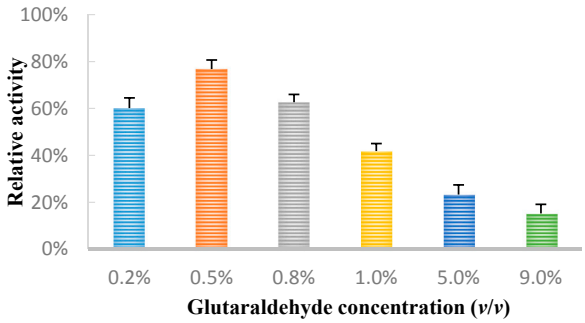

Figure 1. Effect of glutaraldehyde concentration on activity of immobilized glucoamylase. The activity of the free enzyme was 100%. Error bars ± SD.

2.2. Protecting Agents Concentration

A priori, one might expect that the protecting agent affording the most activity will give the best CLEAs. Co-aggregates may contain the enzyme in a less favorable conformation that reverts to a favorable one on re-dissolution. The catalytic center of glucoamylase maintains stability through added protective agents during the cross-linking process; as we would expect, the interior space remains an active site to contend with substrates that may effectively enhance the enzyme activity. As shown in Figure 2a, xanthan gum in the concentration range of 0.5–8.5 mg/g (relative to free glucoamylase) was added to prepare CLEA at pH 5.6. We have observed that the highest activity (86%) was obtained when the xanthan gum was added at 4.5 mg/g, and 18% of the specific activity was enhanced. Its stability was satisfactory. Relative activity of CLEA-XG (xanthan gum) stabilized with the elevation of the protector concentration.

Figure 2b shows that we varied the applied amount of dextrin per milliliter of enzyme solution within a concentration range of 0.5%–3.0% (*v*/*v*). The relative activity of immobilized glucoamylase increased when the amount of dextrin was increased. The obtained specific activity of CLEA-D (dextrin) reached a maximum of 102% at a dextrin concentration of 2.0 wt %, which was approximately 27% of the value previously enhanced with 0.5 wt % protection. This can be explained by the fact that immobilization interferes with the enzyme catalytic site or that diffusional problems are generally produced when immobilized enzymes are acting on macromolecular substrates [14]. Dextrin as a substrate can effectively protect active sites or maintain the enzyme in a catalytically active conformation. During the preparation of CLEA, an immobilized skeleton and porous structure may be formed by cross-linking of protectants; otherwise, enzyme–enzyme and protectant–enzyme co-aggregates formed, which may be an important factor of promoting enzyme activity [27]. However, the poor recyclability and the mechanical strength of CLEA-D decrease with an increase of dextrin, which may affect catalysis and the formation of immobilized enzyme binding with the substrate.

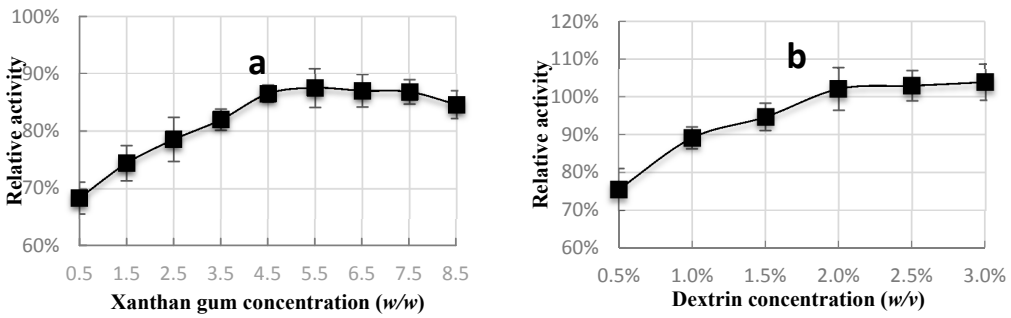

Figure 2. Effect of xanthan gum (**a**) and dextrin concentration (**b**) on activity recovery of prepared cross-linked enzyme aggregates (CLEA). The initial activity of free enzyme was adjudged as 100%. Error bars ± SD.

2.3. Dependence of Catalytic Activity on pH and Temperature

The temperature dependence of the relative activity of the immobilized glucoamylase was compared with that of the free enzyme in Figure 3a. The maximum activities for free glucoamylases, CLEA-XG, and CLEA-D were observed at 60, 60, and 65 °C, respectively. The higher temperature stability was confirmed by the appropriate values of CLEA-D, which has a broader range of tolerance to heat in high-temperature conditions (>55 °C). The relative activity rate of free enzyme was dropping fast at the temperature range 55–75 °C, while the free enzyme shows higher activity at low temperatures.

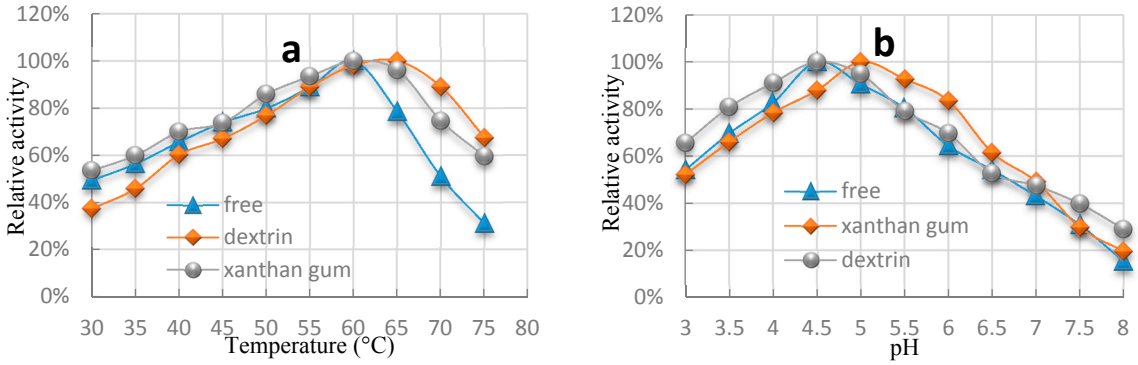

Figure 3. Effect of temperature (**a**) and pH (**b**) on free and immobilized glucoamylase. The maximal activity was adjudged as 100%.

The pH dependence of the relative activity of hydrolysis of soluble dextrin by native and immobilized glucoamylase was measured in the pH range of 3.0–8.0. The optimum pH value was 4.5 for both CLEA-D and free enzyme in Figure 3b; these results are consistent with previous research [28,29]. The broader pH profiles for CLEA-XG may indicate that few conformational changes occur in the enzyme molecule when it combines with xanthan gum.

2.4. Thermostability of Immobilized Glucoamylase

It is well known that the most important characteristic for the industrial application of a biocatalyst is its stability. The thermal stability of glucoamylase more than doubled after immobilization when xanthan gum and dextrin were used as protection. The thermal inactivation of immobilized and free glucoamylase at 60 and 70 °C are investigated in Figure 4. The results show that in the presence of dextrin and xanthan gum the biocatalyst was substantially more stable than the native solution. Figure 4a shows that the inactivation curves of CLEA-D are appreciably higher than CLEA-XG at 60 °C; however, that situation has been dramatically reversed at 70 °C. This interesting stabilization of the immobilized derivative seems to be due to the excellent heat resistance properties of xanthan gum. In this study, the use of dextrin as an active site protector during the cross-linking process increased activity for the modified enzymes at higher temperatures (Figure 4b), which indicates that a greater proportion of the enzyme molecules were thermally stabilized in a favorable conformation.

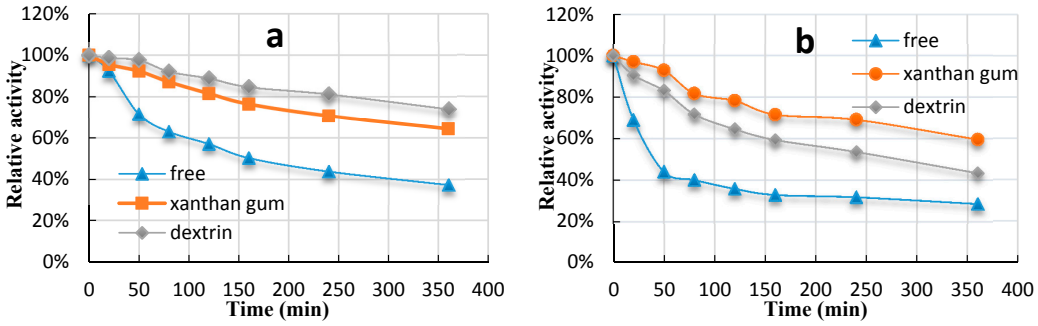

Figure 4. Comparison of thermal stability of free and immobilized glucoamylase, which were incubated at 60 (**a**) and 70 °C (**b**). The initial activity of free and immobilized enzyme was 100%.

The first-order inactivation rate constant (k_i) and inactivation half-time ($t_{1/2}$) of the free and immobilized enzyme were calculated by the thermal inactivation curve (Figure 4). At 70 °C it was found that $t_{1/2}$ of CLEA-XG was almost 10-fold higher than for the soluble enzyme, hinting at possible conformational changes in the molecules of the enzyme–protector complex. Meanwhile, at 60 and 70 °C the vales of k_i for immobilized glucoamylase (Table 1) were higher by one order of magnitude than for the free enzyme. Rigidification occurred as a consequence of multipoint covalent attachment of enzyme molecules by the cross-linking of enzyme molecules by using bifunctional agents.

Table 1. The first-order inactivation rate constant (k_i) and inactivation half-time ($t_{1/2}$) of the free and immobilized enzyme, which were incubated at 60 and 70 °C, respectively. CLEA-XG: cross-linked enzyme aggregates-xanthan gum; CLEA-D: cross-linked enzyme aggregates-dextrin.

Types	60 °C		70 °C	
	k_i (min^{-1})	$t_{1/2}$ (min)	k_i (min^{-1})	$t_{1/2}$ (min)
CLEA-XG	1.49×10^{-4}	463 ± 15	1.67×10^{-3}	415 ± 18
CLEA-D	9.86×10^{-4}	703 ± 21	2.61×10^{-3}	266 ± 14
free	4.33×10^{-3}	160 ± 16	1.65×10^{-2}	42 ± 9

2.5. Kinetic Constants

The kinetic parameters for free and immobilized enzymes were determined by using a Lineweaver–Burk plot (Table 2). The K_m values were a little higher in the case of immobilized enzymes, indicating conformational changes or diffusion limitation of the substrate molecule due to immobilization. CLEA-D showed a higher affinity (K_m = 55 ± 5 μg·L^{-1}) than CLEA-XG (K_m = 70 ± 3 μg·L^{-1}), judging from the K_m value in the case of dextrin as a substrate. The V_m value was very close to that of the free enzyme. Therefore, the native conformation of immobilized glucoamylase is retained almost completely. Effectiveness factor provides information on the role of diffusion in the reaction [30]. In the case of a homogeneous reaction with the native enzyme, η = 1 indicated that hydrolysis was under conditions of complete diffusion. The effectiveness factor values suggest that diffusional effects do not operate in this case. So the method of immobilization was suitable for catalytic reaction of glucoamylase, and the description provides means for the immobilized enzymes.

Table 2. Kinetic parameters and effectiveness factor of native and immobilized glucoamylase.

Types	K_m (μg·L^{-1})	V_m (μg·min^{-1}·mL^{-1})	Effectiveness Factor η
Free	46 ± 3	3.3×10^3	-
CLEA-D	55 ± 5	3.1×10^3	0.94
CLEA-XG	70 ± 3	3.3×10^3	0.99

2.6. Recyclability and Storability

We studied the performance and recyclability of immobilized and free glucoamylase (Figure 5). The CLEA-D had better reusability, as it retained about 45% of the relative activity after 30 runs of repeated use at 60 °C. After 14 times, the decline rate of the activity of CLEA-XG rises by 35%. The lower conversions obtained could be due to mechanical losses of CLEA-XG during the washing and centrifugation procedures. The excellent recyclability of CLEA-D was observed in comparison with CLEA-XG for catalysis of dextrin as a substrate. The effect of immobilization on the storage stability of glucoamylase was investigated by the above method. Both the immobilized and free enzyme retained high activity (more than 70% of initial activity) after two months. However, after one more month, the activity of the free and immobilized enzymes stood at 40% and 60%, respectively, which demonstrated that immobilization slightly increased the storability of glucoamylase.

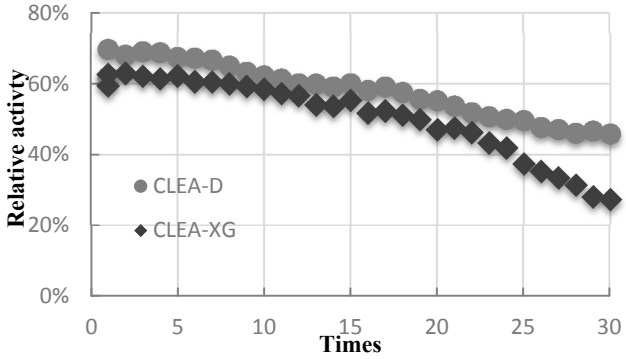

Figure 5. The recyclability of immobilized glucoamylase during 2 wt % dextrin hydrolysis. The immobilized enzymes were stored at 4 °C in a pH 5.5 200 mM sodium acetic buffer. The activity of the free enzyme was taken to be 100%. CLEA-XG: cross-linked enzyme aggregates-xanthan gum; CLEA-D: cross-linked enzyme aggregates-dextrin.

3. Experimental Section

Glucoamylase from *Aspergillus niger* was kindly donated by Enzyme Preparation of Luliang (Yunnan, China). Glutataldehyde solution (50%) and 3.5-Dinitrosalicylic acid (>98%, *w*/*w*) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dextrin and ammonium sulfate were also supplied by Sinopharm Chemical Reagent Co., Ltd. and were of analytical reagent grade. All other reagents were of analytical grade. All the solutions were prepared with distilled deionized water.

3.1. Preparation

3.1.1. Glucoamylase

Glucoamylase (2 g crude enzyme power) was dissolved in 100 mL sodium acetic buffer (200 mM, pH 5.6) in a 200-mL beaker and stirred gently at room temperature for 15 min, which produced a supernatant after centrifugation. To the enzyme solution, the precipitant of $(NH_4)_2SO_4$ solid powder was added, and the mixture was stirred for 20 min. Precipitate was isolated by centrifugation at 5000 rpm at 10 °C for 15 min, then 100 mL sodium acetic buffer (200 mM, pH 5.6) was added and stirred for 15 min at room temperature. Calcium chloride was added to the enzyme solution to remove ammonium sulfate until the final concentration of calcium ions in the solution reached 0.1 M. The enzyme solution was used without any further purification.

3.1.2. Glucoamylase Cross-Linked Enzyme Aggregates (CLEA)

CLEA of glucoamylase was prepared by using a modified procedure from the literature [31,32]. A mixture of 400 μL glucoamylase solution, 600 μL sodium acetic buffer (200 mM, pH 5.6) and 70% (*w*/*w*) powdered $(NH_4)_2SO_4$ (created by adding dextrin power or xanthan gum to prepared CLEA-D or CLEA-XG before precipitation and stirring for 20 min at room temperature) was prepared and aged for 15 min at room temperature with occasional gentle stirring. Next, 50% (*v*/*v*) glutaraldehyde was added to reach a final concentration of 0.5% (*v*/*v*) and aged for 2 h at room temperature with stirring at 300 rpm. The mixture was then diluted with the addition of 1 mL of 200 mM pH 5.6 sodium acetic buffer, vortexed for 15 s, and microfuged for 10 min at 4 °C. The observed dissolving of the enzyme aggregates upon vortexing and microfuging was indicative of incomplete cross-linking of the protein. The supernatant was decanted and the residue was washed three times with sodium acetic buffer (200 mM, pH 5.6), centrifuged, and decanted. The final enzyme preparation was kept in the same buffer at 4 °C. Prior to use, CLEA kept in the buffer was centrifuged and supernatant was decanted.

3.2. Determination and Analysis

3.2.1. Activity Assay

Standard conditions commonly used for the measurement of the activity of soluble and immobilized glucoamylase were as follows. Four hundred microliters of free or equal amounts of immobilized enzyme (the volume used in the process of immobilization) were added to a buffer solution to 600 μL of 2 wt % dextrin gelatinized in water in 0.2 M sodium acetic buffer, pH 5.6. After 10 min of incubation at 60 °C, 3 mL of 3,5-Dinitrosalicylic acid (DNS) was added in order to stop the catalytic reaction. The released glucose was measured by absorbance after 30 min at room temperature and recalculated on the base of glucose standard absorbance. The amount of glucose (in 1 μg) generated for 1 min was used as the unit of enzyme activity (U).

3.2.2. Optimum Temperature, pH, and Kinetic Parameters Assay

Determination of optimum temperature and pH was achieved by individually changing the conditions of the glucoamylase activity assay: temperature ranged from 30 to 80 °C; optimum pH was determined in the citrate (pH 3.0–5.0), phosphate (pH 5.0–7.0), and Tris-HCl (pH 7.0–8.0) buffer. Kinetic parameters (K_m and V_{max} values) were calculated by measuring the initial velocities of the reaction at various substrate concentrations (0.5%–5% (*w*/*v*)); the values were substituted into the Lineweaver–Burk plots to obtain K_m and V_{max}.

3.2.3. Determination of Stability

The thermostability of immobilized and native glucoamylase was measured by incubation at 60 and 70 °C in 0.2 M sodium acetic buffer of pH 5.6 without substrate. They will be taken out in order at times from 0 to 390 min and immediately cooled down in an ice bath. Then the activity of free and immobilized enzyme was determined under the above conditions.

The storage stability of immobilized preparations was estimated by residual enzymatic activity after storage at ambient temperature in the corresponding buffers. The residual activity was periodically measured and compared with the initial activity of the freshly prepared immobilized enzyme (assuming the initial activity of the freshly prepared biocatalyst was 100%).

3.2.4. Recoverability

To evaluate the reusability of immobilized glucoamylase, a series of experiments was carried out under standard assay conditions. The interval of determination was every 3 h and we were sure to rinse the precipitate before each use. The activity of the free enzyme was taken to be 100%.

4. Conclusions

We used a modified CLEA procedure to prepare immobilized glucoamylase with high activity recovery, with added dextrin or xanthan gum as protectors during the cross-linking process. Maltose and maltohexaose were used as active site conformational templates during the harsh cross-linking process [33]. Compared with conventional methods (*i.e.*, covalent or non-covalent combination, embedding, CLEC), the immobilized enzyme and carrier-bound CLEA of glucoamylase with tailor-made properties (e.g., enhanced activity, thermostability, and storage stability) have been designed by a simple and effective technology [31,34–36]. Two types of immobilized enzyme exhibited the expected increase in stability and tolerance compared to the native enzyme. Moreover, the results of storability and recyclability showed that the enzyme has superior performance after immobilization. The recycling experiments suggest that an immobilized enzyme has desirable characteristics and avoids loss of a large amount of activity after 30 uses. Hence, this work has great potential in teaching about immobilization, a relatively new idea for improving the activity of enzymes.

Unfortunately, CLEA exhibited the drawback of inactivation by the generation of steric limitation compared to the free enzyme during the catalysis of large substrates, which perhaps led to fatal contact with the active site of immobilized enzymes [37]. In addition, the enzyme may be inactivated by chemical modification in the process of CLEA preparation; this is due to the fact that the glucoamylase active site often contains lysine and arginine residues, which are potential sites for cross-linking. On cross-linking, the enzyme will be "locked" into this less favorable conformation. For these reasons, to avoid modifying failure, optimization of the CLEA procedure also involves optimization of the cross-linkers/enzyme ratio, precipitants, reaction conditions, *etc.* [38]. The optimum conditions for precipitation and cross-linking were different for glucoamylase from different sources. We could not draw any definite conclusions regarding the influence of the numbers of surface lysine and arginine residues on the activity recovery in CLEA formation and the storage and operational stability of the resulting CLEA. Further studies are going to research the effects of crosslinking on bull serum albumin (BSA), lysine, and the linker regions of the glucoamylase enzyme. Furthermore, to accelerate the industrial application, a feasible way to promote the application was to find a suitable carrier that is combined with CLEA.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. He, J.; Zhang, W.N. Techno-economic evaluation of thermo-chemical biomass-to-ethano. *Appl. Energy* **2011**, *88*, 1224–1232. [CrossRef]
2. Alvira, P.; Tomás-Pejó, E.; Ballesteros, M.; Negro, M.J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.* **2010**, *101*, 4851–4861. [CrossRef] [PubMed]
3. Tester, R.F.; Qi, X.; Karkalas, J. Hydrolysis of native starches with amylases. *Anim. Feed Sci. Technol.* **2006**, *130*, 39–54. [CrossRef]
4. Sheldon, R.A.; van Pelt, S. Enzyme immobilisation in biocatalysis: Why, what and how. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235. [CrossRef] [PubMed]
5. Liese, A.; Hilterhaus, L. Evaluation of immobilized enzymes for industrial applications. *Chem. Soc. Rev.* **2013**, *42*, 6236–6249. [CrossRef] [PubMed]

6. Tardioli, P.W.; Vieira, M.F.; Vieira, A.M.S.; Zanin, G.M.; Betancor, L. Immobilization-stabilization of glucoamylase: Chemical modification of the enzyme surface followed by covalent attachment on highly activated glyoxyl-agarose supports. *Process Biochem.* **2011**, *46*. [CrossRef]
7. Hamerska-Dudra, A.; Bryjak, J.; Trochimczuk, A.W. Immobilization of glucoamylase and trypsin on crosslinked thermosensitive carriers. *Enzym. Microb. Technol.* **2007**, *41*, 197–204. [CrossRef]
8. Sheldon, R.A. Cross-linked enzyme aggregates (CLEAs): Stable and recyclable biocatalysts. *Biochem. Soc. Trans.* **2007**, *35*, 1583–1587. [CrossRef] [PubMed]
9. Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzym. Microb. Technol.* **2007**, *40*, 1451–1463. [CrossRef]
10. Seung, H.S.; Suk, S.C.; Kyungmoon, P.; Young, J.Y. Novel hybrid immobilization of microorganisms and its applications to biological denitrification. *Enzym. Microb. Technol.* **2005**, *37*, 567–573. [CrossRef]
11. Park, J.M.; Kim, M.; Park, J.Y.; Lee, D.H.; Lee, K.H.; Min, J.; Kim, Y.H. Immobilization of the cross-linked *para*-nitrobenzyl esterase of *Bacillus subtilis* aggregates onto magnetic beads. *Process Biochem.* **2010**, *45*, 259–263. [CrossRef]
12. Milosavic, N.; Prodanovic, R.; Jovanovic, S.; Vujcic, Z. Immobilization of glucoamylase via its carbohydrate moiety on macroporouspoly (GMA-*co*-EGDMA). *Enzym. Microb. Technol.* **2007**, *40*, 1422–1426. [CrossRef]
13. Kök, S.; Osman, B.; Kara, A.; Beşirli, N. Vinyl triazole carrying metal-chelated beads for the reversible immobilization of glucoamylase. *J. Appl. Plolym. Sci.* **2011**, *120*, 2563–2570. [CrossRef]
14. Kovalenko, G.A.; Perminova, L.V. Immobilization of glucoamylase by adsorption on carbon supports and its application for heterogeneous hydrolysis of dextrin. *Carbohydr. Res.* **2008**, *343*, 1202–1211. [CrossRef] [PubMed]
15. Bayramoglu, G.; Yilmaz, M.; Arica, M.Y. Immobilization of a thermostable α-amylase onto reactive membranes: Kinetics characterization and application to continuous starch hydrolysis. *Food Chem.* **2004**, *84*, 591–599. [CrossRef]
16. Cao, L.; van Langen, L.; Sheldon, R.A. Immobilised enzymes: Carrier-bound or carrier-free? *Curr. Opin. Biotechnol.* **2003**, *14*, 387–394. [CrossRef]
17. Tischer, W.; Kasche, V. Immobilized enzymes: Crystals or carriers? *Trends Biotechnol.* **1999**, *17*, 326–335. [CrossRef]
18. St Clair, N.L.; Navia, M.A. Cross-linked enzyme crystals as robust biocatalysts. *J. Am. Chem. Soc.* **1992**, *114*, 7314–7316. [CrossRef]
19. Häring, D.; Schreier, P. Cross-linked enzyme crystals. *Curr. Opin. Chem. Biol.* **1999**, *3*, 35–38. [CrossRef]
20. Schoevaart, R.; Wolbers, M.W.; Golubovic, M.; Ottens, M. Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). *Biotechnol. Bioeng.* **2004**, *87*, 754–762. [CrossRef] [PubMed]
21. Wilson, L.; Illanes, A.; Soler, L.; Henrıquez, M.J. Effect of the degree of cross-linking on the properties of different CLEAs of penicillin acylase. *Process Biochem.* **2009**, *44*, 322–326. [CrossRef]
22. Shalviri, A.; Liu, Q.; Abdekhodaie, M.J.; Wu, X.Y. Novel modified starch–xanthan gum hydrogels for controlled drug delivery: Synthesis and characterization. *Carbohydr. Polym.* **2010**, *79*, 898–907. [CrossRef]
23. García-Ochoa, F.; Santos, V.E.; Casas, J.A.; Gómez, E. Xanthan gum: Production, recovery, and properties. *Biotechnol. Adv.* **2000**, *18*, 549–579. [CrossRef]
24. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. *RSC Adv.* **2014**, *4*, 1583–1600. [CrossRef]
25. Aytar, B.S.; Bakir, U. Preparation of cross-linked tyrosinase aggregates. *Process Biochem.* **2008**, *43*, 125–131. [CrossRef]
26. Matijosyte, I.; Arends, I.W.C.E.; de Vries, S.; Sheldon, R.A. Preparation and use of cross-linked enzyme aggregates (CLEAs) of laccases. *J. Mol. Catal. B Enzym.* **2010**, *62*, 142–148. [CrossRef]
27. Shah, S.; Sharma, A.; Gupta, M.N. Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. *Anal. Biochem.* **2006**, *351*, 207–213. [CrossRef] [PubMed]
28. Wang, F.; Guo, C.; Liu, H.Z.; Liu, C.Z. Reversible immobilization of glucoamylase by metal affinity adsorption on magnetic chelator particles. *J. Mol. Catal. B Enzym.* **2007**, *48*, 1–7. [CrossRef]
29. Kumari, A.; Kayastha, A.M. Immobilization of soybean (*Glycine max*) α-amylase onto Chitosan and Amberlite MB-150 beads: Optimization and characterization. *J. Mol. Catal. B Enzym.* **2011**, *69*, 8–14. [CrossRef]

30. Sanjay, G.; Sugunan, S. Glucoamylase immobilized on montmorillonite: Synthesis, characterization and starch hydrolysis activity in a fixed bed reactor. *Catal. Commun.* **2005**, *6*, 525–530. [CrossRef]
31. Mateo, C.; Palomo, J.M.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A. A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. *Biotechnol. Bioeng.* **2004**, *86*, 273–276. [CrossRef] [PubMed]
32. Tao, D.; Lin, Z.; Yu, H.; Xin, T. Preparation of cross-linked aggregates of aminoacylase from *Aspergillus melleus* by using bovine serum albumin as an inert additive. *Bioresour. Technol.* **2010**, *101*, 6569–6571.
33. Germain, P.; Crichton, R.R. Characterization of a chemically modified β-amylase immobilized on porous silica. *J. Chem. Technol. Biotechnol.* **1988**, *41*, 297–315. [CrossRef]
34. Cao, L.Q. Immobilised enzymes: Science or art? *Curr. Opin. Chem. Biol.* **2005**, *9*, 217–226. [CrossRef] [PubMed]
35. Tanriseven, A.; Ölçer, Z. A novel method for the immobilization of glucoamylase onto polyglutaraldehyde-activated gelatin. *Biochem. Eng. J.* **2008**, *39*, 430–434. [CrossRef]
36. Abraham, T.E.; Joseph, J.R.; Bai, L.; Bindhu, V.; Jayakumar, K.K. Crosslinked enzyme crystals of glucoamylase as a potent catalyst for biotransformations. *Carbohydr. Res.* **2004**, *339*, 1099–1104. [CrossRef] [PubMed]
37. Garcia-Galan, C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R.; Rodrigues, R.C. Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv. Synth. Catal.* **2011**, *353*, 2885–2904. [CrossRef]
38. Sangeetha, K.; Abraham, T.E. Preparation and characterization of cross-linked enzyme aggregates (CLEA) of Subtilisin for controlled release applications. *Int. J. Biol. Macromol.* **2008**, *43*, 314–319. [CrossRef] [PubMed]