Research Article

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Simultaneous refolding, purification, and immobilization of recombinant *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase on artificial oil bodies

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Abstract

BACKGROUND: 1,3-1,4-β-D-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.73) has been used in a range of industrial processes. As a biocatalyst, it is better to use immobilized enzymes than free enzymes, therefore, the immobilization of 1,3-1,4-β-D-glucanase was investigated.

RESULTS: A 1,3-1,4-β-D-glucanase gene from *Fibrobacter succinogenes* was overexpressed in *Escherichia coli* as a recombinant protein fused to the N terminus of oleosin, a unique structural protein of seed oil bodies. With the reconstitution of the artificial oil bodies (AOBs), refolding, purification, and immobilization of active 1,3-1,4-β-D-glucanase was accomplished simultaneously. Response surface modeling (RSM), with central composite design (CCD), and regression analysis were successfully applied to determine the optimal temperature and pH conditions of the AOB-immobilized 1,3-1,4-β-D-glucanase. The optimal conditions for the highest immobilized 1,3-1,4-β-D-glucanase activity (7.1 IU mg^{-1} of total protein) were observed at 39 °C and pH 8.8. Furthermore, AOB-immobilized 1,3-1,4-β-D-glucanase retained more than 70% of its initial activity after 120 min at 39 °C, and it was easily and simply recovered from the surface of the solution by brief centrifugation; it could be reused eight times while retaining more than 80% of its activity.

CONCLUSIONS: These results indicate that the AOB-based system is a comparatively simple and effective method for simultaneous refolding, purification, and immobilization of 1,3-1,4-β-D-glucanase.

Keywords: immobilization; *Fibrobacter succinogenes*; 1,3-1,4-β-D-glucanase; artificial oil body

INTRODUCTION

Cellulose is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere. Cellulose biodegradation is very important in several agricultural and waste treatment processes. It could be widely used to produce sustainable biobased products and bioenergy to replace depleting fossil fuels.[1] Cellulose can be degraded by the coordinated action of cellulolytic enzymes, such as endoglucanase, cellobiohydrolase, and β-glucosidase. Endoglucanases cleave glucosidic linkages randomly throughout cellulose molecules, while cellobiohydrolases cleave the disaccharide cellobiose from either the nonreducing or reducing end of the cellulose polymer chain. β-glucosidases hydrolyze cellobiose and low-molecular-mass cellooligosaccharides to release glucose.[2–4] These three types of enzymes act cooperatively to convert cellulose into its constituent sugars. Of these, endoglucanases have received much attention because of their many applications in agricultural, industrial and environmental fields.[5]

1,3-1,4-β-D-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.73) is an endoglucanase that specifically hydrolyzes 1,4-β-D-glucosidic bonds adjacent to 1,3-β-linkages in mix-linked β-glucan, yielding mainly cellobiosyltriose and cellotriosyltetraose.[6] This enzyme has received much attention in both basic and applied research, because of its enzymatic functions and importance in a range of industrial processes, such as supplemental or substitute malt enzymes in beer brewing and increasing the feed conversion efficiency and growth rates of nonruminal animals.[6] If 1,3-1,4-β-D-glucanase could be recycled during the industrial applications, the cost of cellulose conversion would be significantly reduced. Several reports on the immobilization of endoglucanases have been

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published.[7–9] The immobilized endoglucanases were entrapped into alginate or polyacrylamide gels.[7,8] However, immobilization of endoglucanase on the surface of gel beads rather than in beads is important for overcoming diffusion limitations because cellulose, which is the substrate of endoglucanase, is a macromolecule.

A recently established expression/purification system based on artificial oil bodies (AOBs) provides a novel method of enzyme immobilization.[10,11] In this system, oleosin, a unique structural protein of seed oil bodies, is used as the carrier. Oleosin possesses a lipophilic segment embedded into a triacylglycerol core, with two amphipathic arms protruding on the surface of the oil bodies. Thus, the target protein can be expressed in *Escherichia coli* as an insoluble recombinant protein fused to the N- and/or C-terminus of oleosin. Stable artificial oil bodies are then constituted with triacylglycerol, phospholipid and the insoluble oleosin fusion protein. As illustrated previously, the insoluble recombinant proteins fused to oleosin tend to self-refold spontaneously when displayed on AOBs.[12] Thus, this may offer an easy and efficient way to achieve renaturation and immobilization of recombinant proteins in one step.[11]

In the present study, the *F. succinogenes* 1,3-1,4-β-D-glucanase gene was constructed to express as an oleosin-fused protein in *E. coli*. With reconstitution of the AOBs, refolding, purification, and immobilization of the active 1,3-1,4-β-D-glucanase was simultaneously accomplished. Response surface modeling (RSM), with central composite design (CCD), and regression analysis were then employed for the planned statistical optimization of the immobilized 1,3-1,4-β-D-glucanase activity.

MATERIALS AND METHODS

Bacterial culture and DNA manipulation

E. coli BL21 (DE3) (Novagene, Madison, WI) was grown at 37 °C in Luria–Bertani (LB) broth (Difco Laboratories, Detroit, MI). Agar plates were prepared by adding agar (1.5% w/v; Difco) to broth. Plasmid DNA was isolated from *E. coli* using the alkali lysis method.[13] Restriction enzymes and T4 DNA ligase (New England BioLabs Inc., Beverly, MA), were used according to the manufacturer's instructions.

Construction of endoglucanase expression plasmids

The uninterrupted DNA sequence encoding C-terminally truncated *F. succinogenes* 1,3-1,4-β-D-glucanase (GenBank accession number M33676), which has improved enzymatic activity and thermotolerance,[6] was amplified by PCR from the plasmid pNZJ023 using the oligonucleotide forward primer, gluF (5′ CATATGGTTAGCGCAAAGGATTTTAGCGG 3′) and the reverse primer, gluR (5′ GCTCTTCTGCACGATTGCGGAGCAGGTTC 3′).[14] These two primers were designed to place an *Nde*I site at the 5′ end and a *Sap*I site at the 3′ end of the PCR product, respectively. The PCR fragments encoding 1,3-1,4-β-D-glucanase were digested with *Nde*I and *Sap*I, and then ligated with *Nde*I-*Sap*I-digested plasmid pOSP2 to generate pOSP2-glu, which was then sequenced to ensure that no errors were introduced by PCR.[12] The resultant plasmids were used to transform *E. coli* BL21 by standard techniques.[14] Transformants were selected on LB agar plates containing ampicillin (100 μg mL^{-1}) (Sigma Chemical Co., St Louis, MO).

Expression of recombinant proteins

E. coli BL21 transformed cells were cultured in LB broth and cell growth was then measured turbidimetrically at 600 nm (OD_{600}). To produce the recombinant protein, the overnight culture was prepared and subsequently seeded at a 1 : 100 dilution into 5 mL of fresh LB broth. The cell cultures were maintained at 37 °C and induced with 100 μmol L^{-1} of isopropyl-L-D-thiogalactopyranoside (IPTG; Sigma) for protein production upon reaching OD_{600} of 0.5. After 4 h of induction, the cells were harvested by centrifugation at 5000 *g* for 20 min at 4 °C.

Immobilization of 1,3-1,4-β-D-glucanase on AOBs

AOBs were prepared according to the method described by Chiang *et al*.[12] The cell pellet was resuspended in 1 mL of 0.1 mol L^{-1} sodium phosphate buffer (pH 7.4), sonicated for 10 min with an ultrasonicator (Model XL; Misonix, Farmingdale, NY), and fractioned into supernatant and pellet by subsequent centrifugation at 5000 *g* for 20 min at 4 °C. AOBs were reconstituted in 1 mL of 0.1 mol L^{-1} sodium phosphate buffer (pH 7.4) with 15 mg of olive oil (Sigma), 150 μg of phospholipid (Sigma), and the pellet fraction of the *E. coli* cell lysate containing 550 μg of oleosin-fused recombinant proteins. The mixture was subjected to sonication. Subsequently, the reconstituted AOBs were collected after centrifugation and washed with 0.1 mol L^{-1} sodium phosphate buffer solution (pH 7.4). The protein production of each step was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[15]

1,3-1,4-β-D-glucanase activity assays

1,3-1,4-β-D-glucanase activity on the AOBs was determined using barley β-glucan (Megazyme International, Wicklow, Ireland) as the substrate. Reducing sugar released from the substrate was estimated using the dinitrosalicylic acid (DNS) method.[16] One unit of enzyme activity was defined as that releasing 1 μmol of reducing sugar equivalents per minute from the substrate under the assay conditions, with specific activity expressed as IU mg^{-1} protein. Protein concentration was determined using Lowry assay against a standard curve of bovine serum albumin, fraction V (Sigma).[17]

Optimum pH and temperature of immobilized 1,3-1,4-β-D-glucanase

RSM, its main effects and the interaction between the different factors each at level, was simultaneously investigated. CCD with two variables at five levels and five replicates at the central point, for a total of 13 experiments were conducted. In this experimental design, pH (X_1) and temperature (X_2) were chosen as factors, with units of 1,3-1,4-β-D-glucanase activity denoted by Y. According to preliminary experimental results, the experimental index number, scaled and real values are shown in Table 1. The scaled values were defined as follows: $X_1 = (\text{pH} - 9)$; $X_2 = (\text{T} - 40)/10$. The experimental design, data analysis and regression model building were performed using Design Expert (version 5.07, Stat-Ease Inc., Minneapolis, MN) software. The responses, as linear, quadratic and cubic functions of the variables, were tested for adequacy and fitness using analysis of variance (ANOVA). Model analysis and the lack-of-fit test were used for selection of adequacy models. A model with P-values ($P > F$) less than 0.05 was regarded as significant. The highest-order significant polynomial was selected. The lack-of-fit test was used to compare the residual and pure errors at replicated design points. The response predictor was discarded where lack-of-fit was significant, as indicated by a low probability value ($P > F$). The model with no significant lack-of-fit was selected. Predicted residual sum of the squares (PRESS) was used as a measure of the fit of the model to the points in the design. The smaller the PRESS statistic is, the better the model fits the data points.[18]

Table 1. Process variables used in the ccd, showing the treatment combinations and the mean experimental responses for AOB-immobilized 1,3-1,4-β-D-glucanase

Treatment	Coded setting levels (X_1 = pH; $X_2 = T$) X_1	X_2	Actual levels (X_1 = pH; $X_2 = T$) X_1	X_2	1,3-1,4-β-D-glucanase activity[a] (IU mg^{-1} of total protein)
1	−1	−1	8	30	3.5
2	0	−1.41	9	25.9	2.88
3	−1.41	0	7.59	40	5.93
4	0	0	9	40	6.93
5	1	1	10	50	0.4
6	0	0	9	40	6.81
7	0	0	9	40	6.77
8	1.41	0	10.41	40	0.306
9	0	0	9	40	6.56
10	1	−1	10	30	2.39
11	0	1.41	9	54.1	2.58
12	0	0	9	40	6.73
13	−1	1	8	50	4.48

[a] Results represent the mean of three experiments.

After the optimal conditions for the immobilized 1,3-1,4-β-D-glucanase activity were predicted, a series of experiments were conducted in triplicate and repeated three times in order to check the reliability of the RSM with the predicted values and experimental data. The results were analyzed using ANOVA test available from the Statistical Analysis System software (SAS; version 8.1; Statistical Analysis System Institute, Cary, NC). Duncan's multiple range test was used to detect differences between the predicted and observed values.[19]

Thermal stability of immobilized 1,3-1,4-β-D-glucanase

The thermal stability of AOB-immobilized 1,3-1,4-β-D-glucanase was determined by incubation at 39, 50 and 60 °C in 0.1 mol L^{-1} Tris-HCl buffer (pH 8.8) containing 0.5% barley β-glucan (Megazyme) as the substrate. Aliquots were withdrawn at intervals of 0, 20, 40, 60, 80, 100, and 120 min, and the residual enzyme activity was measured.

Reusability of immobilized 1,3-1,4-β-D-glucanase

The reusability of AOB-immobilized 1,3-1,4-β-D-glucanase was determined by incubation at 39 °C for 20 min in 0.1 mol L^{-1} Tris-HCl buffer (pH 8.8) containing 0.5% barley β-glucan (Megazyme) as the substrate. At the end of the reaction, the immobilized enzyme was recovered by centrifugation and the supernatant was removed for further analysis. The reaction was reinitiated by administration of the recovered enzymes in a fresh substrate solution.

RESULTS AND DISCUSSION

Simultaneous refolding, purification, and immobilization of 1,3-1,4-β-D-glucanase on AOBs

The DNA fragments encoding C-terminally truncated *F. succinogenes* 1,3-1,4-β-D-glucanase were amplified by PCR and subcloned into the AOB expression/purification system vector pOSP2. 1,3-1,4-β-D-glucanase was overexpressed in *E. coli* as a recombinant protein fused to the N terminus of oleosin by a linker polypeptide. After induction with IPTG at 37 °C, the induced and non-induced recombinant bacteria containing the 1,3-1,4-β-D-glucanase genes were analyzed using SDS-PAGE. A band of about 65 kDa corresponding to the 1,3-1,4-β-D-glucanase-oleosin fusion protein was observed in the induced recombinant bacteria (Fig. 1, lane 2). The 1,3-1,4-β-D-glucanase-oleosin fusion protein was found predominately in the insoluble fraction of the cell lysate after centrifugation (Fig. 1, lane 4). After reconstitution into AOBs, the 1,3-1,4-β-D-glucanase-oleosin fusion protein, as well as other insoluble bacterial proteins, was almost entirely present in the oil-body fraction after centrifugation (Fig. 1, lane 6). The DNS method was then used to confirm 1,3-1,4-β-D-glucanase activity of the reconstituted AOBs. Detectable levels of 1,3-1,4-β-D-glucanase activity were observed in the reconstituted AOBs from the induced recombinant bacteria, indicating that 1,3-1,4-β-D-glucanase was immobilized on the AOB surface and folded into the active structure. In addition, the yield of the reconstituted AOBs was 9.44±1.25 mL, starting from 1.5 g (wet weight) of *E. coli* cells, while the protein concentration of the reconstituted AOBs was 0.16 ± 0.02 µg µL^{-1}.

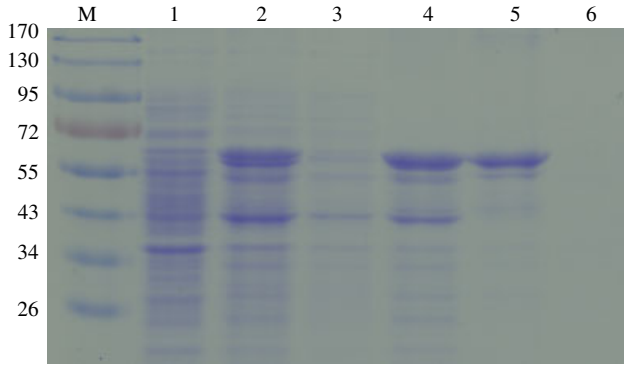

Figure 1. SDS-PAGE analysis of 1,3-1,4-β-D-glucanase immobilized on AOBs. *Lane M*, molecular weight marker; *lane 1*, cell lysate of the recombinant *E. coli* before IPTG induction; *lane 2*, cell lysate of the recombinant *E. coli* after IPTG induction; *lane 3*, soluble fraction of the cell lysate after centrifugation; *lane 4*, insoluble fraction of the cell lysate after centrifugation; *lane 5*, AOBs; *lane 6*, remaining supernatant after AOBs recovery.

Although the cost of commercial cellulolytic enzyme preparations has been reduced significantly in recent years, enzyme costs are still an obstacle to full-scale process commercialization.[20] Immobilization on an inert carrier offers the prospect of significant cost savings by facilitating enzyme recycling through multiple cycles of batch-wise hydrolysis. Additionally, enzyme immobilization frequently results in improved thermal stability or resistance to shear inactivation.[21] In the past, immobilized endoglucanases were usually entrapped into alginate or polyacrylamide gel beads.[7,8] However, immobilization of endoglucanase on the surface of gel beads rather than in beads is important for overcoming diffusion limitations because the macromolecular property of the substrate cellulose restricts passage through the bead matrix. In the current study, 1,3-1,4-β-D-glucanase was fused to the N terminus of oleosin and then mixed with plant oil to form AOBs. The enzyme activity assay confirmed the presence of active 1,3-1,4-β-D-glucanase on the surfaces of the AOBs. Compared with conventional immobilization methods,

Table 2. Model analysis (a), lack-of-fit (b), and *R*-squared analysis (c) for measured responses

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-value	*F*-value in statistic table
(a) Model analysis[a]					
mean	243.53	1	243.53		
linear	21.85	2	10.93	2.12	0.1710
quadratic	50.41	3	16.80	99.92	<0.0001**
cubic	1.00	2	0.50	13.89	0.0091**
residual	0.18	5	0.036		
total	316.96	13	24.38		
(b) Lack-of-fit[b]					
linear	51.51	6	8.59	474.32	<0.0001**
quadratic	1.10	3	0.37	20.34	0.0069**
cubic	0.11	1	0.11	5.92	0.0717
pure error	0.072	4	0.018		

(c) *R*-squared analysis[c]

	R-squared	PRESS
Linear	0.2976	83.22
Quadratic	0.9840	7.97
Cubic	0.9976	6.97

** Statistically significant at 99% of confidence level.
[a] Model analysis: select the highest order polynomial where the additional terms are significant.
[b] Lack-of-fit: want the selected model to have insignificant lack-of-fit.
[c] *R*-squared analysis: focus on the model minimizing the PRESS.

the use of AOBs as immobilization matrices reduces the requirement for 1,3-1,4-β-D-glucanase purification and eliminates the cost of immobilization. In particular, 1,3-1,4-β-D-glucanase immobilized on AOBs can be recovered easily from the surface of the solution simply by a brief centrifugation. These results prove that AOB immobilization is a comparatively simple and effective method of refolding, purification, and immobilization of 1,3-1,4-β-D-glucanase.

Optimization of immobilized 1,3-1,4-β-D-glucanase activity

Temperature and pH were identified as the major factors affecting the activity of 1,3-1,4-β-D-glucanase.[6] RSM is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results. CCD, which minimizes the number of experimental runs, was used to determine the effects of independent variables on the dependent variables. The process variables used in the experimental design and results for enzyme activities are shown in Table 1. Treatments 4, 6, 7, 9 and 12 (central points) showed the highest levels of 1,3-1,4-β-D-glucanase activity.

Table 2 compares the validities of the linear, quadratic and cubic models for the responses according to their *F*-values. The cubic model appeared to be the most accurate, with a statistically insignificant lack-of-fit ($P > 0.05$) and the smallest PRESS value (6.97). In addition, the goodness of fit of the cubic model was checked using the coefficient of determination ($R^2 = 0.9976$), indicating that just 0.24% of the total variation was not explained by the model. This confirms that the accuracy and general ability of the cubic model was good, and analysis of the associated response trends was reasonable.

Table 3. Coefficient estimates by regression modeling

Factor	Coefficient estimate	Standard error	*P*-value
intercept	6.76	0.085	
pH*	−0.61	0.210	0.0353
T	−0.40	0.210	0.1185
pH × pH**	−1.88	0.072	<0.0001
T × *T***	−2.07	0.072	<0.0001
pH × *T***	−0.74	0.095	0.0005
pH × pH × pH**	−0.69	0.130	0.0036
T × *T* × *T*	0.15	0.130	0.3243

* Statistically significant at 95% of confidence level.
** Statistically significant at 99% of confidence level.

The cubic model generated by the design is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2 + \beta_{111} {X_1}^3 + \beta_{222} {X_2}^3 + \varepsilon$$

where β_0 is constant, β_1 and β_2 are the main effect of each process variable (pH and temperature), β_{11} and β_{22} are effect of the square of the variables, β_{12} is the interaction effect between the variables, β_{111} and β_{222} denote the effect of the cube of the variables, Y is endoglucanase activity, X_1 and X_2 represent the independent variables (pH and temperature), and ε is the random error.

The RSM yielded the following regression equation:

$$Y = 6.76 - 0.61\text{pH} - 0.40T - 1.88\text{pH}^2 - 2.07T^2 - 0.74\text{pH} \times T - 0.69\text{pH}^3 + 0.15T^3$$

where Y is the predicted response for 1,3-1,4-β-D-glucanase activity (IU mg^{-1} of total protein), and pH and T are the coded values for pH and temperature (see Table 1).

The significance of the coefficients determined by Student's *t*-test and the related *P*-values are presented in Table 3. The latter were used to check the significance of each coefficient, and also indicated the strength of the interaction between each independent variable (i.e. the smaller the *P* value, the more significant the corresponding coefficient).[22] In this study, the *P* value of pH, second-order pH and T, the interaction coefficient of pH and T, and third-order pH were significant ($P < 0.05$). The high significance of the pH second- and third-order model indicates that it can act as a limiting factor, with even small variations substantially altering 1,3-1,4-β-D-glucanase activity.[23] The model also clearly reveals significant interactions between pH and T ($P < 0.0001$), therefore, treating them separately may not reflect their real influence on the 1,3-1,4-β-D-glucanase activity (e.g. optimum pH activity changes along with T).

The RSM for enzyme activity as a function of pH and temperature of the immobilized 1,3-1,4-β-D-glucanase is depicted in Fig. 2. The results indicate that the optimal conditions for the immobilized 1,3-1,4-β-D-glucanase activity occur at 39 °C and pH 8.8. To confirm the applicability of the model, 1,3-1,4-β-D-glucanase activity at the suggested optimum conditions was determined. Under these conditions, the model predicted enzymatic activity of 6.9 IU mg^{-1} at a confidence level of 95%. The experimental enzymatic activity of 7.1 IU mg^{-1} confirmed the accuracy of the model.

1,3-1,4-β-D-glucanases are important industrial enzymes due to their potential application in a range of industrial processes.

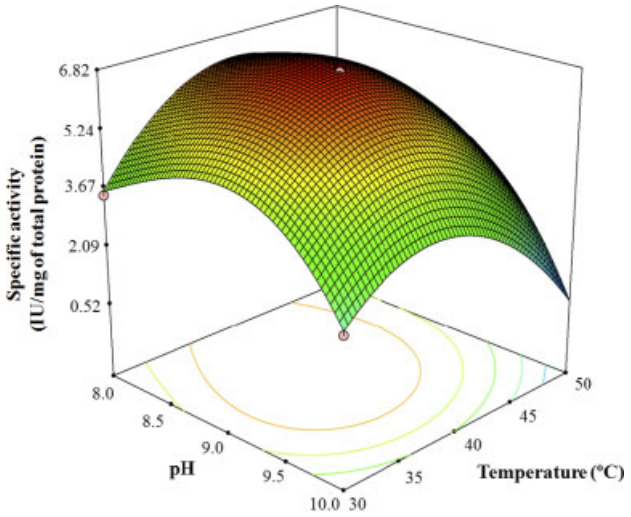

Figure 2. Response surface plot for the effects of pH and temperature on AOB-immobilized 1,3-1,4-β-D-glucanase activity.

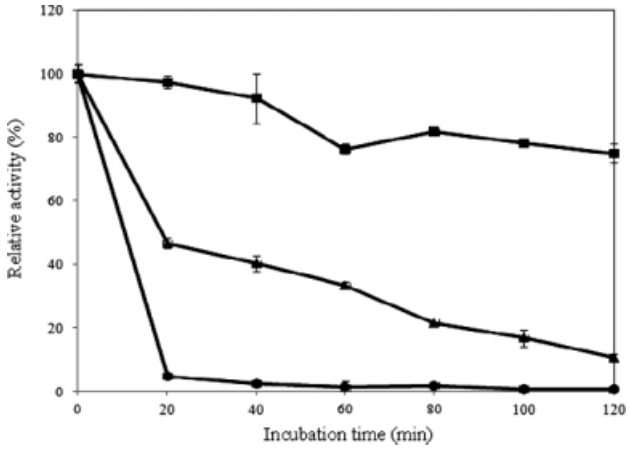

Figure 3. Thermal stability of AOB-immobilized 1,3-1,4-β-D-glucanase incubated at 39 °C (■), 50 °C (▲), or 60 °C (●) for 120 min in 0.1 mol L^{-1} Tris-HCl buffer (pH 8.8) containing 0.5% barley β-glucan. The bars represent standard errors of the means of triplicate measurement.

However, a major drawback to their widespread use as industrial enzymes is their thermal stability during industrial processes. This has spurred considerable research into the production of more thermally stable variants.[6] The 1,3-1,4-β-D-glucanase used in this study is a C-terminally truncated form of *F. succinogenes* 1,3-1,4-β-D-glucanase, which has improved enzymatic activity and thermotolerance. Wen *et al*.[6] previously determined that the optimum temperature and pH of the free C-terminally truncated 1,3-1,4-β-D-glucanase was 50 °C and between pH 6 and 8, respectively. However, the interactions between pH and temperature on the C-terminally truncated *F. succinogenes* 1,3-1,4-β-D-glucanase activity have never previously been evaluated. In this study, the maximum activity of immobilized 1,3-1,4-β-D-glucanase was determined at 39 °C and pH 8.8. Therefore, immobilization of the 1,3-1,4-β-D-glucanase on AOBs slightly increased the optimum pH but decreased the optimum temperature of the 1,3-1,4-β-D-glucanase activity.

Thermal stability of immobilized 1,3-1,4-β-D-glucanase

Thermal stability is a very important issue when considering the industrial applications of enzymes. In this study, the immobilized 1,3-1,4-β-D-glucanase retained more than 70% of its initial activity after 120 min of heating at 39 °C (Fig. 3). According to Wen *et al*., the activity of free C-terminally truncated *F. succinogenes* 1,3-1,4-β-D-glucanase was reduced to 50% of maximum after 10 min at 50 °C.[6] In this study, AOB-immobilized 1,3-1,4-β-D-glucanase still retained more than 60% of its activity after 10 min of heating at 50 °C (Fig. 3). Thus, better thermal stability was demonstrated for the immobilized 1,3-1,4-β-D-glucanase compared with the free enzyme. Chiang *et al*.[11] immobilized *Agrobacterium radiobacter* D-hydantoinase on AOBs and also demonstrated that the immobilized enzyme exhibited a higher thermal tolerance than the free enzyme. In addition, Li *et al*.[24] immobilized cellulase on the outer membrane of liposomes and indicated that the liposome membrane was favorable for maintaining the activity of the cellulase in these cellulase-containing liposomes. Therefore, we suggest that the interaction between enzyme and oil-body membrane is beneficial for reducing enzyme deactivation.

Reusability of immobilized 1,3-1,4-β-D-glucanase

Immobilized enzymes are preferred in industrial applications because in this form, they can be recycled and, thus, reduce production costs. AOBs can be separated from the reaction mixture by flotation centrifugation, thus the 1,3-1,4-β-D-glucanase immobilized on AOBs could be recovered and recycled. To evaluate this possibility, the recovery and operational stability of AOB-immobilized 1,3-1,4-β-D-glucanase was examined during eight successive rounds of β-glucan hydrolysis. The AOB-immobilized 1,3-1,4-β-D-glucanase was reused for six cycles at 39 °C without loss of activity. After that, the enzyme activity gradually declined, but still retained more than 80% of its initial activity after eight cycles of enzyme use (Fig. 4). It is interesting to note that because 1,3-1,4-β-D-glucanase immobilized on AOBs floats rather than sinks, it is much easier to recover in heterogeneous mixtures containing precipitable solids, which could confound attempts to recover enzymes bound to solid matrices. However, the operational stability of enzymes must be evaluated because it is one of the most important factors affecting the success of the applications of an immobilized system. In this study, results prove that 1,3-1,4-β-D-glucanase immobilized on AOBs under optimal conditions can be reused many times, thereby reducing the cost of enzymes used in industrial processes where the cost of the enzyme is significant.

CONCLUSION

The gene encoding 1,3-1,4-β-D-glucanase from *F. succinogenes* was cloned and expressed as an oleosin-fused protein in *E. coli*. Simultaneous refolding, purification, and immobilization of the active 1,3-1,4-β-D-glucanase was accomplished as a result of reconstituting AOBs, and RSM with CCD and regression analysis were successfully applied to determine the optimal temperature and pH conditions for AOB-immobilized 1,3-1,4-β-D-glucanase. The optimal conditions for immobilized 1,3-1,4-β-D-glucanase activity were observed at 39 °C and pH 8.8. In addition, the AOB-immobilized 1,3-1,4-β-D-glucanase was reused eight times while retaining more than 80% of its activity. It was recovered easily from the surface of the solution by brief centrifugation. These results prove that an AOB-based system is a comparatively simple

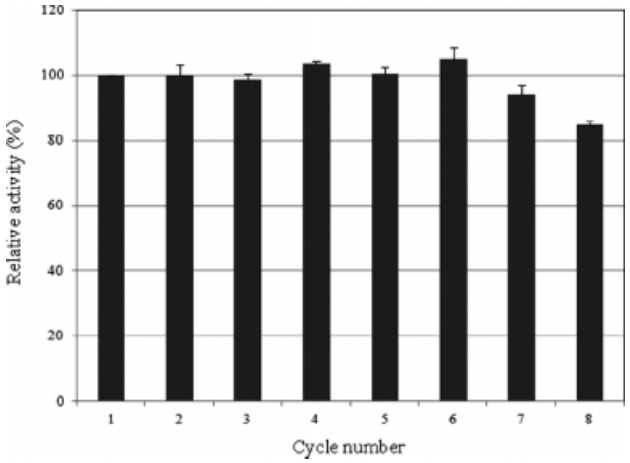

Figure 4. Reusability of the AOB-immobilized 1,3-1,4-β-D-glucanase incubated at 39 °C for 20 min in 0.1 mol L^{-1} Tris-HCl buffer (pH 8.8) containing 0.5% barley β-glucan. The bars represent the standard errors of the means of triplicate measurement.

and effective method for simultaneous refolding, purification, and immobilization of 1,3-1,4-β-D-glucanase.

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