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Immobilization of Alternansucrase on Chitosan Beads

Kusuma Thongplaew[1], Thanapon Charoenwongpaiboon[1], Karan Wangpaiboon[1], Rath Pichyangkura[1], and Manchumas Prousoontorn[1*]

[1]Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phayathai Rd, Wangmai, Pathumwan, Bangkok 10330, Thailand

**Corresponding author. E-mail: manchumas.h@chula.ac.th*
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ABSTRACT

Alternansucrase (Alt, EC 2.4.1.140) is an enzyme that catalyzes transferring of an α-D-glucosyl residue from sucrose to another sucrose molecule which acts as an acceptor, thereby creating a glucan with alternating α-1,6 and α-1,3-glycosidic linkages, known as alternan. The truncated *alt* (ΔC-*alt*) gene was expressed in *E. coli* BL21(DE3) and recombinant Alt was partially purified by DEAE anion exchange chromatography. The recombinant Alt possessed an optimum temperature and pH of 40°C and 4.0, respectively. In order to reuse the enzyme and to enhance its stability, Alt was immobilized on a chitosan support by covalent method using glutaraldehyde as a crosslinking agent. Chitosan beads were activated under optimal immobilization condition which was 0.5% (w/v) glutaraldehyde in 50 mM citrate buffer pH 6.0 at 4°C overnight. After that, the activated chitosan beads were incubated with an enzyme solution (30 U or 0.69 mg/g of beads) at 4°C overnight. The immobilized enzyme will be further employed for the production of alternan.

Keywords: Immobilization, alternansucrase, chitosan beads, alternan

INTRODUCTION

Alternansucrase (Alt, EC 2.4.1.140) is a bacterial enzyme belonging to Glycoside hydrolase family 70 (GH70). It catalyzes the transferring of glucosyl units from sucrose to another sucrose molecule which acts as an acceptor to form a long chain polymer named alternan (Cote et al., 1982). Alternan is an α-D-glucan consisting of alternating α-1,3 and α-1,6 linkages. Due to its unusual alternating linkage structure, it is apparently resistant to several microbial and mammalian hydrolytic enzymes, but can only be cleaved by isomaltodextranases and alternanases. Alternan can also be used as an ingredient/ substitution in various industries such as gum arabic substitutes, noncaloric bulking agents, prebiotic dietary supplements, and papermaking aids (Cote et al., 1997).

Since the immobilization of Alt has never been reported so far, we herein are interested in the immobilization of the enzyme on a chitosan bead. Immobilization technique has some advantages as follow; improving stability of the enzyme against environmental conditions, facilitating enzyme isolation from the reaction medium, reducing production costs, and the possibility of reusing the enzyme in continuous processes (Datta et al., 2013; Sedaghat et al., 2009). For repeated use of Alt in successive batches, the enzyme can be prepared in an immobilized form.

Natural polymers like chitosan have widely been used as supports for immobilization. Chitosan is a natural cationic polysaccharide, which is a copolymer formed by units of 2-deoxy-N-acetyl-D-glucosamine and 2-deoxy-D-glucosamine linked by β-1,4 glycosidic bonds. It rarely occurs in nature but can generally be obtained by alkaline and by enzymatic deacetylation of chitin. Chitosan is biocompatible, biodegradable, and nontoxic, making it very attractive for various applications (Campos et al., 2013). Glutaraldehyde has frequently been used as a cross-linking agent, as it is less expensive, readily available, and highly soluble in aqueous solution. The high reactivity of the aldehyde groups, which readily form imine bonds (Schiff's base) with amino groups and acetal bonds with hydroxyl groups, provides the high efficiency of glutaraldehyde on the cross-linking of chitosan (Campos et al., 2013).

In this work, we described the expression and biochemical characterization of Alt from the truncated a*lt* (ΔC-*alt*) gene. We immobilize Alt on a chitosan support by covalent method then using glutaraldehyde as a crosslinking agent. Biochemical characterization of both free and immobilized enzymes were then explored.

MATERIALS AND METHODS

Expression of truncated *alt* (ΔC-*alt*) gene in *E. coli* and production of recombinant Alt

The recombinant plasmid (pETD7) was transformed into *E. coli* BL21(DE3). The transformants were grown in LB broth containing 100 mg/L of ampicillin at 37°C until OD_{600} reached 0.6. The cell cultures were then induced with 0.4 mM of IPTG at 16°C for 20 hrs. The culture medium and cells were separated by centrifugation at 8,000 × g for 10 min. The cell pellets were re-suspended in 0.3 mL of buffer per 1 mL of culture volume, containing 50 mM sodium citrate buffer pH 6.2, 75 mM of sodium chloride, and 0.1% (v/v) Triton X-100. The cells were lysed by sonication, and the cell lysate was fractionated by centrifugation at 10,000 × g, 4°C for 10 min. The crude supernatant containing the enzyme was collected and stored at 4°C for further analysis (Wangpaiboon et al., 2018).

Purification of Alt

The crude Alt was pumped through Sartorius Vivaflow 50 modular cross-flow system, containing an inbuilt ultrafiltration membrane with 100,000 MWCO and applied to a DEAE-Toyopearl-650 M (Tosoh Bioscience) column (Wangpaiboon et al., 2018). The fraction exhibiting enzymatic activity were pooled and analyzed on 8% SDS-PAGE gel.

Alt enzymatic activity assay

Enzymatic reaction containing 222 mU of purified enzyme (5.2 µg) with 500 mM sucrose and 50 mM citrate buffer pH 5.0 in 0.5 mL total volume were incubated at 40°C for 10 min. The reaction was stopped by adding 0.5 mL of 3,5-dinitrosalicylic acid (DNS) then boiled for 10 min. The A_{540} was measured (Miller, 1959). One unit of Alt activity was defined as the amount of the enzyme that released 1 µmole of fructose per minute, using fructose as a standard.

Effect of substrate concentration on Alt activity

The activity of Alt was carried out in a 0.5 mL reaction volume using the following conditions: 0 to 500 mM sucrose, 50 mM sodium citrate buffer pH 5.0, 40°C, and 222 mU of purified enzyme (5.2 µg) was used for these analyzes.

Effect of temperature and pH on Alt activity

The activity of Alt was assayed at various incubation temperatures between 20-80°C in 50 mM citrate buffer pH 4.0. The effect of pH was analyzed in 50 mM of citrate buffer pH 3.0 – 6.0 and phosphate buffer pH 6.0-8.0 at 40°C (Wangpaiboon et al., 2018). A 222 mU of purified enzyme (5.2 μg) was used for these analyses. Relative activity at different temperatures and pH were determined by the DNS method.

Preparation of chitosan beads

Twenty grams of chitosan was dissolved in 1 L of a mixed acid solution containing 2% (w/v) of acetic acid, 1% (w/v) of lactic acid, and 1% (w/v) of citric acid. The resulting chitosan solution was introduced dropwise into 0.8 N NaOH by a peristaltic pump. The resultant chitosan beads were washed with deionized water until pH became neutral (Charoenwongpaiboon et al., 2018).

Immobilization of Alt on chitosan beads

The immobilization conditions, such as glutaraldehyde and enzyme concentrations, were optimized. Chitosan beads were activated with 0.1–3.0% (w/v) glutaraldehyde in a citrate buffer with a pH 6.0 at 4°C overnight. After that, the activated chitosan beads were incubated with an enzyme solution (10–80 U/g of beads; or 0.23–1.84 mg/g of beads) at 4°C overnight with mild agitation. The immobilized enzyme was washed with 1 M NaCl to remove an electrostatically adsorbed enzyme, then was washed with 0.1% (v/v) Triton X-100 to remove any hydrophobic adsorbed enzyme and was washed 3 times with 50 mM citrate buffer pH 4.0. The resultant immobilized enzyme was kept at 4°C until further use. The immobilized activity and activity yield served as parameters to optimize immobilization conditions. The immobilized activity was calculated as immobilized activity (U)/g of beads and the activity yield (%) was expressed as activity on beads (U)/ [initial enzyme (U)–unbound enzyme (U)] × 100 (Charoenwongpaiboon et al., 2018).

RESULTS AND DISCUSSION

Expression and purification of Alt

The 160 kDa of molecular weight of Alt was successfully expressed in *E. coli* BL21 (DE3) with specific activity of 42.70 U/mg of protein. The recombinant Alt was partially purified by anion exchange chromatography. The percent recovery of Alt was 66.96% of the total activity with 2.28 purification fold (Table 1). The purified enzyme exhibited a high degree of apparent purity on SDS-PAGE, shown as a major single band in Fig. 1

Table 1. The purification table of Alt

Step	Fraction volume (mL)	Total protein (mg)	Activity (units)	Specific activity (unit/mg)	fold	%Yield
Crude enzyme	300.0	255	4776	18.73	1	100
Ion-exchange chromatography	144.0	74.9	3198	42.70	2.28	66.96

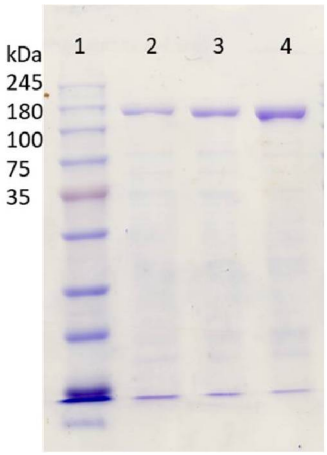

Figure 1. SDS-PAGE analysis of purified Alt. Lane 1: protein molecular weight marker, lane 2: crude enzyme (1.69 mg protein), lane 3 and 4, purified protein from Viva Flow 50 (1.69 mg protein) and a DEAE-Toyopearl column (1.69 mg protein).

Effect of substrate concentration on Alt activity

The effect of substrate concentration (0-500 mM sucrose) on Alt activity was studied (Fig. 2). The results showed that the Alt activity increased with an increase in sucrose concentration. Nevertheless, when sucrose concentration was higher than 100 mM, the activity did not increase. So, the concentration of sucrose at 100 mM was chosen.

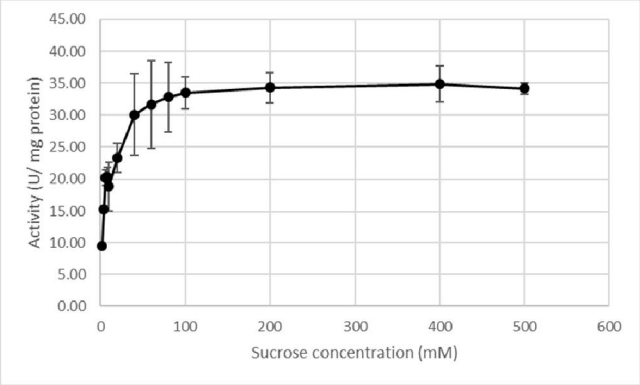

Figure 2. Effect of substrate concentration on Alt activity. The data represent means of three assays and error bars represent the standard deviation of three experiments.

Effect of temperature and pH on Alt activity

Alt showed an optimal pH of 4.0 (Fig. 3A), and an optimum temperature of 40°C (Fig. 3B). The results were corresponded to previous report where the optimal activity was as well found to be at pH 4.0 and 40°C (Wangpaiboon et al., 2018).

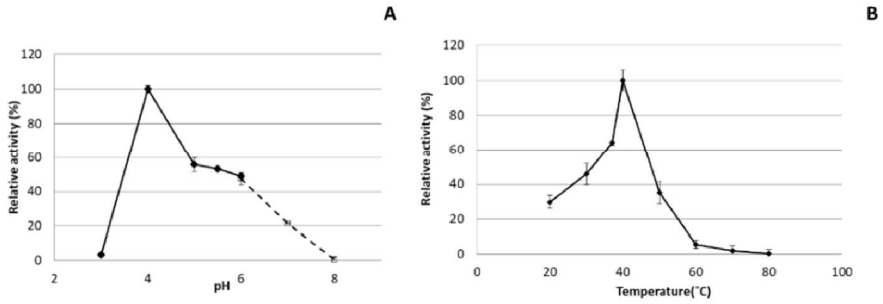

Figure 3. Biochemical characterization of Alt. (A) Effect of pH on activity of Alt (—◆—, citrate buffer; - ▣ -, phosphate buffer). (B) Effect of temperature on the activity of Alt. The data represent means of three assays and error bars represent the standard deviation of three experiments.

Immobilization of Alt on chitosan beads

The purified Alt was immobilized on chitosan beads via glutaraldehyde. The immobilization involves two steps: the first is activation of amino-functionalized beads using glutaraldehyde in 50 mM citrate buffer pH 6.0 at 4°C for overnight. The second is covalent attachment of Alt. The immobilization conditions such as glutaraldehyde and enzyme concentrations were optimized.

The effect of glutaraldehyde concentration (0.1–3.0%, w/v) on the immobilization procedure was then investigated at pH 6.0 (Fig. 4). The results showed that the immobilized activity and activity yield increased with an increase in glutaraldehyde concentration. Nevertheless, when glutaraldehyde concentration was higher than 0.5% (w/v), the immobilized activity did not increase. This finding indicated that the increase in glutaraldehyde concentration resulted in more covalent bonds per enzyme molecule and therefore may cause a conformational change of the enzyme. In addition, at a higher concentration of glutaraldehyde, there is good chance of a covalent modification close to the active site of the enzyme leading to enzyme inactivation.

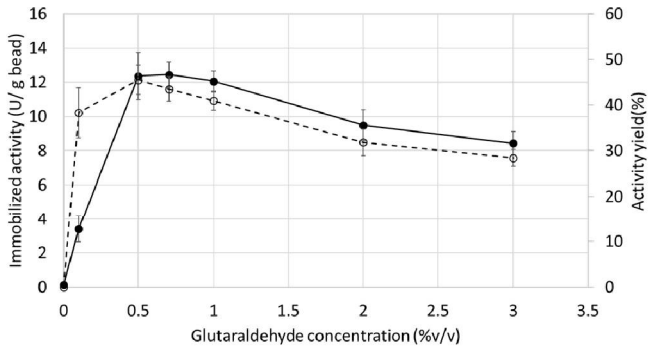

Figure 4. Effects of glutaraldehyde concentrations on the immobilized activity (—●—) and activity yield (- ⊖ -) of immobilized Alt. The data represent means of three assays and error bars represent the standard deviation of three experiments.

The effect of enzyme concentration on enzyme immobilization was examined by incubating activated chitosan beads (0.5% [w/v] glutaraldehyde), with different concentrations of the enzyme (Fig. 5). When the amount of Alt added per gram of beads increased from 10 to 30 U/ g bead, the activity immobilized enzyme rapidly increased. After that, the activity of immobilized enzyme reached a plateau and the activity yield was found to decrease. This result might be explained as the reactive groups on the support were saturated with the enzyme. For further analysis, an enzyme concentration of 30 U/g was chosen because it provided the high activity yield of approximately 59.7%. The immobilized enzyme was washed with NaCl to remove an electrostatically adsorbed enzyme, then the immobilized enzyme was washed with triton X-100 serve as detergent to remove any hydrophobic adsorbed enzyme.

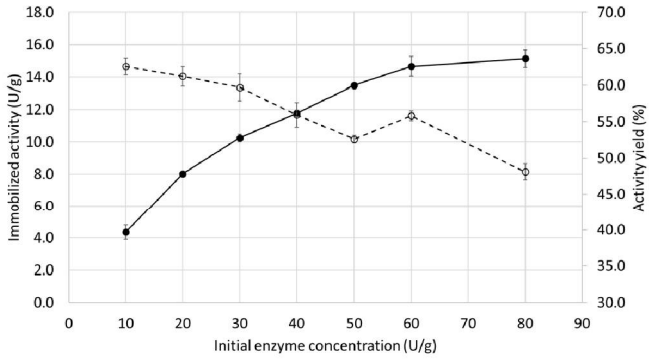

Figure 5. Effects of enzyme concentrations on the immobilized activity (-●-) and activity yield (-⊖-) of immobilized Alt. The data represent means of three assays and error bars represent the standard deviation of three experiments.

CONCLUSION

The ΔC-*alt* gene was successfully expressed in *E. coli*. The recombinant Alt possessed an optimum temperature and pH of 40 °C and 4.0, respectively. The immobilization conditions such as glutaraldehyde and enzyme concentrations were optimized. The chitosan beads were activated through the reaction between amino groups and glutaraldehyde for Alt immobilization. The optimal immobilization conditions were to use glutaraldehyde concentration of 0.5% (w/v) and enzyme concentration of 30 U/g. The obtained immobilized enzyme will then be further used for the production of alternan.

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