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Research Article

TRANSGLYCOSYLATION ACTIVITY AND CHARACTERIZATION OF RECOMBINANT SUCROSE PHOSPHORYLASE FROM *LEUCONOSTOC MESENTEROIDES* MBFWRS-3(1) EXPRESSED IN *ESCHERICHIA COLI*

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ABSTRACT

Objective: Sucrose phosphorylase (SPase) is an enzyme that catalyzes the transfer of glucosyl to various acceptor molecules. Distinct types of SPases have been reported, and their transglycosylase activities have been shown to differ. In general, glycosylation is a process that is used to modify bioactive compounds. As such, glycosylation can increase the chemical stability of compounds and improve their characteristics such as reduce strong smell and sour taste. We previously cloned recombinant SPase (SPaseWRS-3[1]) from *Leuconostoc mesenteroides* MBFWRS-3[1] in *Escherichia coli*. In the current study, we aimed to characterize SPaseWRS-3 and determine its transglycosylation activity using benzoic acid (BA), ascorbic acid, and kojic acid (KA).

Methods: Expression analyses were conducted in lysogeny broth (LB) medium supplemented with tetracycline and expression was induced using isopropyl-β-d-thiogalactopyranoside. The characteristics of the 56 kDa recombinant SPase (rec-SPase) were confirmed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Rec-SPase activity was determined spectrophotometrically using sucrose as the substrate and NADPH as the end-product at 340 nm. Transglycosylation activity was evaluated using thin-layer chromatography (TLC) on silica gel plates.

Results: Our results demonstrated that the rec-SPase had an activity of 98.52% relative to the reference SPase (ref-SPase). BA and KA were determined to undergo glucosyl transfer by rec-SPase using ref-SPase, as observed with TLC. Our findings are consistent with those reported previously for the SPase isolated from *L. mesenteroides*.

Conclusion: Recombinant SPase activity is comparable to reference SPase activity. Our study could be the initial study to deeply observe SPase activity in other substrates as well.

Keywords: *Escherichia coli*, Kojic acid, Benzoic acid, Ascorbic acid, *Leuconostoc mesenteroides*, Sucrose phosphorylase.

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INTRODUCTION

Sucrose phosphorylase (SPase) is an enzyme that catalyzes glycosylation reactions such as the reversible catalysis of sucrose and inorganic phosphate to α-D-glucose-1-phosphate (G-1-P) and D-fructose, a reaction that has been widely utilized in industry for decades [1]. In this reaction, the SPase enzyme transfers the glucosyl section of G-1-P and sucrose to various acceptor molecules, which are used in the manufacture of both food and cosmetic materials. For example, these biologically active compounds can be used as drug precursors and/or converters of other biologically active compounds to improve their characteristics. As such, glycosylation can neutralize the occurrence of a strong smell, sour taste, or low solubility [2,3].

SPases have been found in several bacteria, including lactic acid bacteria (LAB) classified as generally recognized as safe such as *Bifidobacterium lactis* [4,5], *Leuconostoc mesenteroides* [6-8], *Bifidobacterium longum* [9,10], and *Oenococcus oeni* [11]. Recently, SPase has become the focus of increasing research interest due to its ability to generate intermediate drug precursors. Despite the broad acceptor specificity of the enzyme, differences in enzyme specificities among bacterial species have been reported.

The activity of SPase from *L. mesenteroides* has been used in a variety of industrial applications, including to transfer the glucosyl moiety of G-1-P to various sugars and sugar alcohols [12], to transfer the glucosyl moiety of sucrose to phenolic or alcoholic hydroxide groups of various substances [12-16], and to transfer the glucosyl moiety of sucrose or of G-1-P to acceptors such as galactose, maltose, and glucose or beta linkage compounds such as cellobiose and gentiobiose using sucrose as a glucosyl donor to produce various acceptor reaction products [17]. Because LAB is considered safe for humans, the production of enzymes in some species for food- and health-related industrial applications is highly promising. For example, the formation of 2-O-a-D-glucopyranosyl-L-ascorbic acid (AA-2G) from AA plays an important role in maintaining skin elasticity and in repairing damaged skin compared with AA [18]. Moreover, the derivative of this enzyme sucrose 6^F-phosphate phosphorylase has provided a novel insight into the human gut microbiome [19].

In the previous studies [20], we screened, identified, and cloned the SPase gene from *L. mesenteroides* MBFWRS-3(1), which was isolated from a sugar-containing beverage from Solo, Indonesia. SPaseWRS-3(1) from the indigenous Indonesian LAB was found to have a 99% amino acid sequence similarity to an existing enzyme, 1355SPase [20]. Accordingly, this previous study provided valuable information on the molecular diversity of bacterial SPases, particularly with respect to their unique transglycosylation characteristics and the SPase recombinants.

In this study, we aimed to produce recombinant SPaseWRS-3(1) on a large scale and study the transglycosylation activity of recombinant SPaseWRS-3(1) using the substrates benzoic acid (BA) and AA. In addition, rec-SPase activity was measured using sucrose as a substrate, with the measurement of NADPH as the end-product at 340 nm.

Transglycosylation activity assays were conducted using thin-layer chromatography (TLC) with acetonitrile + water and butanol + acetic acid + water as the mobile phase on silica gel plates. Our results revealed that the best glucosyl transfer reaction activities of rec-SPase and ref-SPase were obtained using BA (Rf = 0.55), which was able to produce lower Rf (0.15–0.2) substances, as observed on the TLC plate.

MATERIALS AND METHODS

Materials

The SPase gene was cloned from *L. mesenteroides* MBFWRS-3(1) into the plasmid pAM-SPWRS-3(1) followed by transformation into *Escherichia coli* BL-21 Star™ (pAM_SPaseWRS3), as previously described [20].

Protein expression and purification of recombinant SPase

A single colony of *E. coli* BL-21 Star™, carrying the recombinant plasmid pAM_SPaseWRS-3(1), was inoculated into 5 mL of LB medium containing 5 µg/mL tetracycline and incubated overnight under shaking conditions at 37°C. The culture was then inoculated into 500 mL of LB medium until the OD_{600} reached 0.2. Subsequently, 500 µL of 5 µg/mL tetracycline was added and the culture was incubated with shaking at 200 rpm at 37°C for 1 h. Protein expression was induced by the addition of 500 µL 1 mM isopropyl-β-d-thiogalactopyranoside (Wako, Japan) followed by additional incubation at 37°C with shaking. Fermentation was continued at 30°C for 2 h. The cells were then collected by centrifugation (15,000× *g*, 10 min, 4°C), suspended in phosphate buffer (50 mM, pH 6.8), and disrupted by ultrasonication for 15 min (Branson Sonifier, England). The enzyme was harvested by centrifugation (6000× *g* at 4°C for 10 min).

Ni-NTA affinity chromatography was then used to purify 6× His-tagged recombinant SPase (1 mL, His SpinTrap; GE Healthcare, Germany). The purified C-terminal 12-histidyl tagged protein was collected by elution with 500 mM of imidazole buffer (20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole at a pH of 7.4) at a flow rate of 1 mL/min. The molecular mass of the recombinant SPaseWRS-3(1) obtained was determined using the Laemmli system (Laemmli 1990) using a 10% acrylamide (BioRad, USA) gel. Proteins were stained with Coomassie Blue R-250 (BioRad).

Enzyme activity assay and protein content quantification

SPase activity was measured using a modified method of Silverstein [1,18]. Briefly, the standard assay involved the usage of 60 mM potassium phosphate buffer (pH 6.4), 0.14 M sucrose, 0.09 mM EDTA-Na_2, 0.36 mM $NADP^+$, 0.003 mM d-glucose-1,6-diphosphate, 15 mM $MgCl_2$, 6 units α-phosphoglucomutase mL^{-1}, and 6 units glucose-6-phosphate dehydrogenase mL^{-1}. The reaction mixture was then incubated under five different pH conditions (pH 5, 6, 7, 7.5, and 8) at five different temperatures (25°C, 30°C, 35°C, 37°C, and 40°C). The increase in absorbance was measured using a spectrophotometer at 340 nm.

One unit of SPase activity was defined as the amount of enzyme that released 1 µmol $NADP^+$ min^{-1}. An SPase standard (0.5 units) from *L. mesenteroides* (Oriental Yeast, Co., Ltd., Japan) was used as the positive control, whereas sterile water was used as the negative control. Protein concentration was measured according to the Bradford method (1976), with bovine serum albumin (Fermentas, USA) as the standard.

Transglycosylation activity assay with BA, AA, and kojic acid (KA)

Transglucosylation of BA was conducted in water containing 0.4% BA and 20% sucrose at pH 5.1. Two units of SPase were added to the mixture and the reaction mixture was incubated at 37°C for 16 h. The reaction product was then analyzed by TLC using acetonitrile + water (70:30) as the mobile phase on silica gel plates. The spot resulting from TLC was detected at 254 nm [3].

Transglucosylation of AA was performed in 100 mM HEPES buffer at pH 7.5 containing 0.5% (w/v) AA and 30% (w/v) sucrose. The reaction mixture containing 20 µL of HEPES buffer and 0.2 unit/µL SPase was then incubated at 37°C for 15 h. The reaction was stopped by the elimination of SPase with an Ultrafree-MC (10,000 NMWL, Millipore, USA). The reaction product was analyzed by TLC using butanol + acetic acid + water (3:1:1) as the mobile phase on silica gel plates [2].

Transglucosylation of KA was performed with a reaction volume of 10 mL at pH 7.5; the reaction mixture contained 0.4 units of recombinant SPaseWRS-3(1), 200 µg KA (Nacalai Tesque, Japan), 0.1 M phosphate buffer, and 30% sucrose. The reaction was incubated at 37°C for 7, 8, 12, and 24 h. The reaction was stopped by the elimination of SPase with Centricon (10,000 NMWL cut-off, Millipore, USA). The reaction product was analyzed using TLC-densitometry with acetonitrile and dH_2O (80:20) as the mobile phase and silica gel plates as the solid phase. Peaks were detected at an absorbance of 254 nm by a TLC scanner.

RESULTS AND DISCUSSION

Recombinant SPaseWRS-3(1) protein confirmation

We evaluated the overexpression of recombinant SPase from *E. coli* BL-21 Star™, and purification of the protein was achieved using 12-histidyl residues, which were fused to the C-terminal end of the protein during construction. The purified SPases from the *E. coli* lysates appeared as a dominant single band slightly <66 kDa (Fig. 1) on SDS-PAGE and with Coomassie Brilliant Blue staining. This is consistent with the predicted molecular mass of 57 kDa that has been reported previously [20]. After purification and desalting, it was determined that the BL-21 Star™ recombinant could be produced at a high yield and exhibited high activity compared with ref-SPase (98% of the SPase reference activity), as shown in Fig. 2. The optimum temperature for activity was 37°C, whereas the optimum pH was found to be 7, with an SPase recombinant concentration of 1117.5 µg/mL.

SPase from *L. mesenteroides* has been researched for several decades [6,7]. Although these SPases are produced from the same species (*L. mesenteroides*), the C-terminal region sequences of *L. mesenteroides* SPases have been found to be different depending upon the source. It is assumed that the diversity in sucrose-related genes and their products in different bacteria is responsible for this.

Although three potential catalytic amino acid residues, namely, Asp-196, Glu-237, and Asp-295, are located in the conserved sequences of the *L. mesenteroides* SPases, there exists some dissent on the diversity of the transglycosylation properties of SPases with respect to the acceptors and their products [2,17,21,22]. This variability could be due to the involvement of the C-terminal region. Thus, different protein structures may exist, as indicated by different transglycosylation yields and acceptor efficiencies.

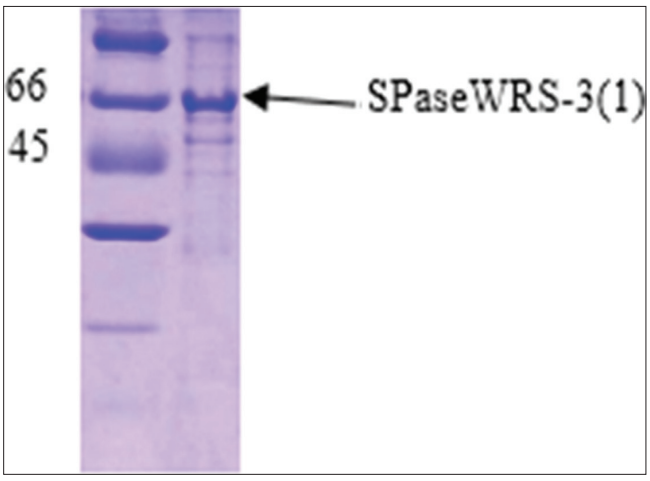

Fig. 1: SDS-PAGE analysis of purified recombinant sucrose phosphorylase produced by *Escherichia coli* BL21 STAR™

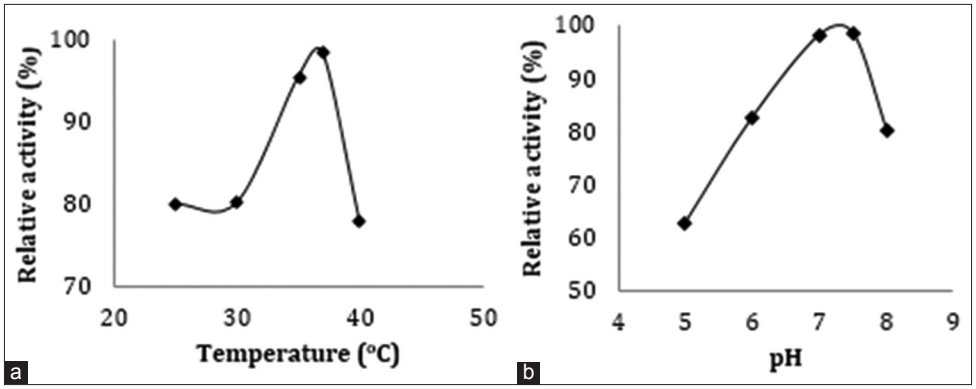

Fig. 2: Relative activity of SPaseWRS-3(1) against the SPase reference at different temperatures (a) and pH values (b)

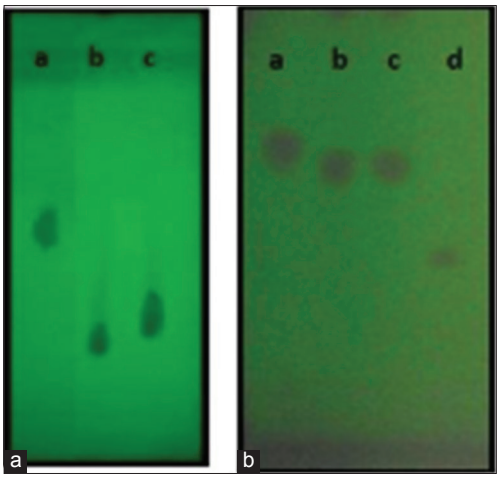

Fig. 3: Thin-layer chromatography analysis of benzoic acid (BA) (Rf 0.55). (a), Reaction of BA with the SPase standard (Rf 0.15); (b), reaction of BA with the SPase recombinant (Rf 0.2); (c), acetonitrile + water (70:30) as the mobile phase on silica gel plates A. TLC analysis of ascorbic acid (AA) (Rf 0.75) (a), reaction of AA with SPase standard (Rf 0.72); (b), reaction of AA with SPase recombinant (Rf 0.72); (c), AA2G (Rf 0.5); (d) using butanol + acetic acid + water (3:1:1) as the mobile phase on silica gel plates B

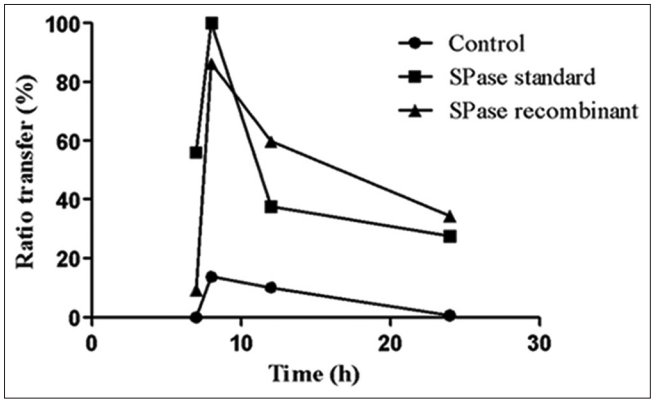

Fig. 4: Ratio of the transfer of kojic acid over time

The strain of *L. mesenteroides*, from which the SPase is sourced, may affect the structure of the gene and lead to different yields. The strain MBFWRS-3(1) was isolated from Wedang Ronde, a popular hot Javanese dessert containing glutinous rice balls stuffed with peanut paste floating in a hot sweet ginger and lemongrass tea. In this case, the sample was obtained from the liquid portion, which contains a mixture of brown and regular sugar. The strain MBFWRS-3(1) was then confirmed as *L. mesenteroides* by 16S rDNA analysis [23].

Transglycosylation activity

Transglycosylation activity assays demonstrated that the highest glucosyl transfer reaction activities of rec-SPase and ref-SPase were obtained using BA (Rf 0.55), which produced lower Rf (0.15–0.2) substances, as observed on the TLC plates (Fig. 3a). In contrast, AA did not show activity because the Rf values of the reactions of AA with the SPase standard or with the recombinant enzyme did not differ (Fig. 3b). For KA, transglycosylation activity was shown because there was movement of the substances based on the Rf values. However, the separation was minimal.

The conversion of KA to KA transglycosylate increased over time over the 8 h period (Fig. 4). Subsequently, the reaction reversed, producing KA after 24 h. These results are in line with those of previous studies because the transglycosylation reaction was reversible [12,18,2]. The differences in the transglycosylation activity of KA with SPase-rec, which has a ratio probability of only 86%, maybe due to the diversity of the SPase enzyme. Indeed, Malik *et al*. (2011) studied amino acid residue variations in SPase-rec (SPaseWRS-3(1)) from *L. mesenteroides* and found differences of approximately 16% [20]. This variability likely impacts the protein structure, resulting in decreased affinity for substrates and changes in enzyme flexibility. Collectively, these factors are believed to underlie the diversity of the enzyme.

SPase from *L. mesenteroides* has shown transglycosylation activity with BA, particularly under acidic conditions [24]. Moreover, SPase from *L. mesenteroides* reportedly cannot transfer the glucosyl moiety of sucrose to BA at pH 7.5 [10]. However, the enzyme is reported to catalyze the transglucosylation reaction to BA at pH 5 [24]. The results of these studies are consistent with those of the current study, which showed the presence of transglycosylation activity when analyzed using TLC.

To stabilize AA, particularly under thermal and oxidative conditions, the derivative AA2G is produced through transglycosylation activity. This new substance is extremely stable *in vitro* and exhibits AA activity *in vivo* after enzymatic hydrolysis to AA by α-glucosidase [25,26]. During the process of AA phosphorolysis, SPase catalyzes the transfer of the glucosyl moiety of sucrose to G-1-P and D-fructose, a reaction which is reversible [12]. The transglucosylation activity of AA has previously been demonstrated by Kwon *et al*. (2007) using SPase-rec from *B. longum*. They detected the major product using HPLC and confirmed the presence of AA2G using LC-MS/MS [2]. However, the results of our study did not reveal any product from this enzymatic reaction. This is likely because the rec-SPase used in the current study was produced from *L. mesenteroides* and had different properties compared with the enzymes derived from other bacteria. Moreover, another previous study has demonstrated than an optimum pH of 5.2 could selectively catalyze

SPase to convert sucrose to AA2G with high efficiency and perfect selectivity in *B. longum* [27]. Our results are consistent with those of a previous study conducted by Kitao and Sekine (1992) in which they did not find an AA glucosylation reaction.

CONCLUSION

In this study, we successfully produced recombinant SPase on a large scale from *E. coli* BL-21 Star™, with a molecular weight of approximately 45–66 kDa and relative activity of approximately 98%. The activity of recombinant SPase is comparable to that of reference SPase. The best glucosyl transfer reaction activities of rec-SPase and ref-SPase were achieved using BA (Rf=0.55), which produced low Rf (0.15–0.2) substances, as observed by TLC. Additional quantitative assays using HPLC and BA to ensure the activity of the transglucosylation product are warranted. Our study could be the initial study to deeply observe SPase activity in other substrates as well.

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CONFLICTS OF INTEREST STATEMENT

All authors declare no conflicts of interest to declare in this project.

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