INTRODUCTION

Sucrose phosphorylase (SPase) is an enzyme that catalyzes the transfer of glucosyl to various acceptor molecules. Different 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 MBFWS-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 (IPTG). The characteristics of the 56 kDa recombinant SPase (rec-SPase) were confirmed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The location of the enzyme was determined 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 glycosyl 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.
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 glucose 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-SPaseWRS-3(1) following 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 tetacycline and incubated overnight under shaking conditions at 37°C. The culture was then inoculated into 500 mL of LB medium until the OD600 reached 0.2. Subsequently, 500 μL of 5 μg/mL tetacycline 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), resuspended 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 Brilliant 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-Na2, 0.36 mM NADP+, 0.003 mM d-glucose-1,6-diphosphate, 15 mM MgCl2, 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)

Transglycosylation 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 the reaction product was then analyzed by TLC using acetonitrile + water as the mobile phase/on silica gel plates.

Transglycosylation 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 Centrifor (10,000 NMWL, Millipore, USA). The reaction product was analyzed using TLC densitometry with acetonitrile and dH2O (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 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 (90% 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 117.5 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 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.

Transglycosylation 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 contained 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 SPases with 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.
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The strain of \textit{L. mesenteroides}, from which the SPase is sourced, may affect the structure of the gene and lead to different yields. The strain MBFQRS-3(1) was isolated from Wedang Ronde, a popular 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 MBFQRS-3(1) was then confirmed as \textit{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 \textit{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 \textit{L. mesenteroides} has shown transglycosylation activity with BA, particularly under acidic conditions [24]. Moreover, SPase from \textit{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 \(\alpha\)-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 \textit{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 \textit{L. mesenteroides} and had different properties compared with the enzymes derived from other bacteria. Moreover, another previous study has demonstrated that 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 Kita 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* BI-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=0.5), 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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