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Transglucosylation of caffeic acid by a recombinant sucrose phosphorylase in aqueous buffer and aqueous-supercritical CO_2 media

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ABSTRACT

Polyphenolic compounds, such as caffeic acid, offer many health benefits, but their industrial applications are limited because of their low solubility in water and instability under UV light or heat. In this study, we enzymatically produced caffeic acid glucosides, in both aqueous buffer and aqueous-supercritical carbon dioxide (SC-CO_2) media, using a recombinant sucrose phosphorylase (SPase) from *Bifidobacterium longum*. By using LC/MS/MS analysis, we verified that the enzymatic reaction products were caffeic acid monoglucosides and diglucosides. Under SC-CO_2, the amounts of the reaction products from the enzyme reaction were smaller than those in the aqueous reaction medium. However, this is the first report of the transglucosylation of caffeic acid by SPase, and also the first enzymatic reaction with phenolic compounds conducted in a SC-CO_2 phase.

1. Introduction

Polyphenolic compounds are important bioactive substances that are prevalent in edible plants. Among them, caffeic acid [3-(3,4-dihydroxyphenyl)-2-propenoic acid] is one of the most abundant hydroxyl cinnamic acids, and is found naturally in various agricultural products, such as fruit, vegetables, wine, olive oil and coffee beans (Moon & Terao, 1998). Since caffeic acid is known to possess important and strong physiological and antioxidative activities (Laranjinha, Vieira, Madeira, & Almeida, 1995; Nardini, Natella, Gentili, DiFelice, & Scaccini, 1997), its application to functional foods and cosmetics has increased extensively. However, such applications are limited by the low water solubility and instability of polyphenolic compounds. Enzymatic modification of phenolic acids (such as adding glucosides by transglycosylation) has generated interest as a mechanism to side-step these hurdles, since such changes can increase aqueous solubility and chemical stability of polyphenolic compounds (Bae et al., 2002; Bertrand et al., 2006; Moon, Kim, et al., 2006; Moon, Lee, et al., 2006).

Sucrose phosphorylase (SPase), which is listed as sucrose: phosphate α-D-glucosyltransferase [EC 2. 4. 1. 7] by the Enzyme Commission, is known to reversibly catalyse the following reaction: sucrose + phosphate = D-fructose + α-D-glucose 1- phosphate (G 1-P) (Silverstein, Voet, Reed, & Abeles, 1967). SPase has broad acceptor specificity, and transfers the glucosyl moiety of sucrose to phenolic or alcoholic OH groups of various substances (Kitao, Ariga, Matsudo, & Sekine, 1993; Kitao, Matsudo, Saitoh, Horiuchi, & Sekine, 1995). Due to the broad acceptor specificity of SPase from *Leuconostoc mesenteroides* and *Bifidobacterium longum*, it has been applied to many substances with base units of gallic acid or flavone, such as catechins (Kitao et al., 1993, 1995), hydroquinone (Kitao & Sekine, 1994; Kwon & Lee, 2004), hydrofuranones (Kitao, Matsudo, Sasaki, Koga, & Kawamura, 2000) and ascorbic acid (Kwon, Kim, & Lee, 2007). However, for derivatives of cinnamic acid, such as caffeic acid, which itself has many derivatives, e.g. coumaric acid and ferulic acid, known as strong antioxidants, there has been no report on transglucosylation using SPase.

When conducting transglucosylation of polyphenolic compounds in aqueous media, low substrate solubility in water impedes reactions in an aqueous buffer, and low reaction yields are unavoidable. Enzymatic reactions with polyphenolic compounds are, therefore, commonly conducted in aqueous methanol solutions, since polyphenolics have higher solubility in methanol. From an industrial point of view, however, if the product is edible or pharmaceutical in nature and the solvent possesses toxicity, the necessary product purification and solvent recovery demand a higher operating cost and substantial effort. Meanwhile, for enzymatic reactions that involve lipids, such as transesterification, previous studies have extensively considered organic solvents and

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supercritical carbon dioxide (SC-CO_2) as alternate reaction media (Hammond, Karel, Klibanov, & Krukonis, 1985; Randolph, Blanch, Prausnitz, & Wilke, 1985; Zaks & Klibanov, 1988). SC-CO_2, the hydrophobic CO_2 existing beyond its critical point (31.0 °C and 73.8 bar), shows particular potential as an ideal reaction medium for lipid transesterification, due to its low toxicity and the ease of solvent recovery. Currently, there are no reports that exploit SC-CO_2 as a medium for glucose-transferring reactions in polyphenols.

In this study, we employed aqueous and aqueous- SC-CO_2 media to perform transglucosylation of caffeic acid using a recombinant transglucosyltransferase, SPase, cloned into *Escherichia coli* from *B. longum*. Using LC/MS/MS, we subsequently verified that the transglucosylation reaction, in both media, produced caffeic acid glucosides.

2. Materials and methods

2.1. Materials

All chemicals used in this study, including caffeic acid, sucrose, methanol and potassium phosphate, were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.2. Transglucosylation of caffeic acid in aqueous buffer and aqueous-SC-CO_2 media

B. longum SPase, produced in recombinant *E. coli* JM109, as previously described (Kim et al., 2003; Shin, Jung, Lee, Kim, & Kim, 2008), was used for the transglucosylation reaction. One unit (U) of enzyme activity was defined as the amount of enzyme that liberate 1 μmol of fructose from the substrate in one min (Van den Broek et al., 2004). Caffeic acid and its derivatives in an enzymatic reaction mixture were assayed by Kitao's method (Kitao et al., 1993). To transglucosylate caffeic acid in an aqueous medium, a reaction mixture with a total volume of 1.0 ml, containing sucrose (30.0%, w/v), caffeic acid (1.0%, w/v) and SPase (18 U) in 50 mM potassium phosphate buffer (pH 6.8), was incubated in a shaking water bath at 100 rpm at 42 °C for 5 to 15 h. In an aqueous-SC-CO_2 medium, a reaction mixture was incubated at 150, 200 or 250 bar at 42 °C for 5 or 10 h. After reactions were completed, 20 μl of each reaction mixture were subjected to HPLC analysis.

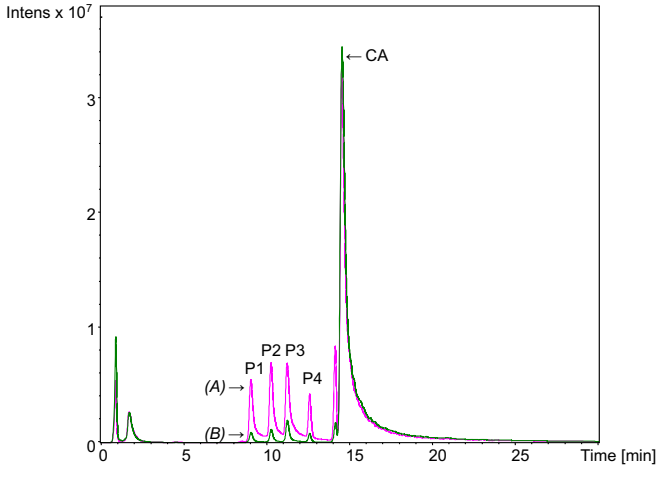

Fig. 1. Total ion chromatogram of reaction products of caffeic acid (CA) and sucrose by the recombinant SPase from *Bifidobacterium longum* in (A) aqueous medium and (B) aqueous-SC-CO_2 medium. The peaks P3 and P4 were considered as transfer products, and the peaks P1 and P2 were by-products from the SPase reaction.

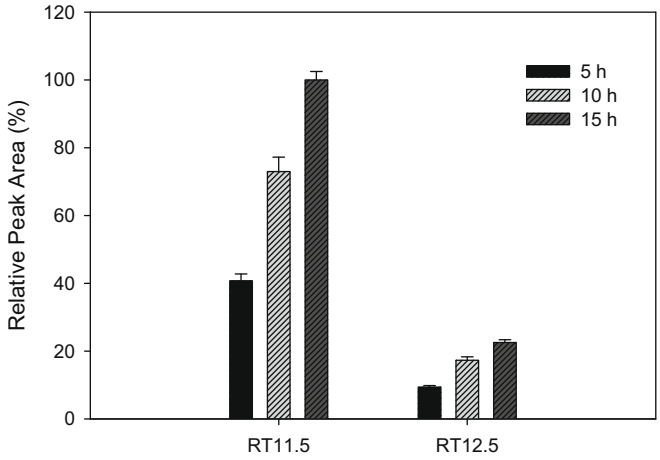

Fig. 2. Production of caffeic acid glucosides by SPase in aqueous medium at 42 °C. RT 11.5 and RT 12.5 represent the retention times of the peaks corresponding to caffeic acid diglucoside and monoglucoside, respectively.

2.3. HPLC analysis of the reaction mixtures

Enzyme reaction mixtures were analysed by an Agilent 1100 system (Agilent Technologies; Waldbronn, Germany) equipped with a UV detector set at 280 nm, and a column of Hypersil ODS (Thermo; Waltham, MA, USA). The mobile phase was switched from acetic acid solution (0.5%, v/v) to acetonitrile solution (30% in 0.5% acetic acid, v/v), using a gradient elution at a flow rate of 1.0 ml/min for 27 min at 20 °C.

2.4. LC/MS/MS analysis

Caffeic acid glucoside was analysed by HPLC coupled to an ion trap mass spectrometer (Agilent Technologies) with electrospray ionisation (ESI) in both ESI-MS and ESI-MS/MS modes, operated by LC/MSD Trap software (V5.2; Bruker Daltonik GmbH; Germany). Each sample was injected through the HPLC into the ESI source at a flow rate of 0.8 ml/min. The capillary voltage of the ion source was set at +3500 V for negative ion mode acquisitions. The flow rate of

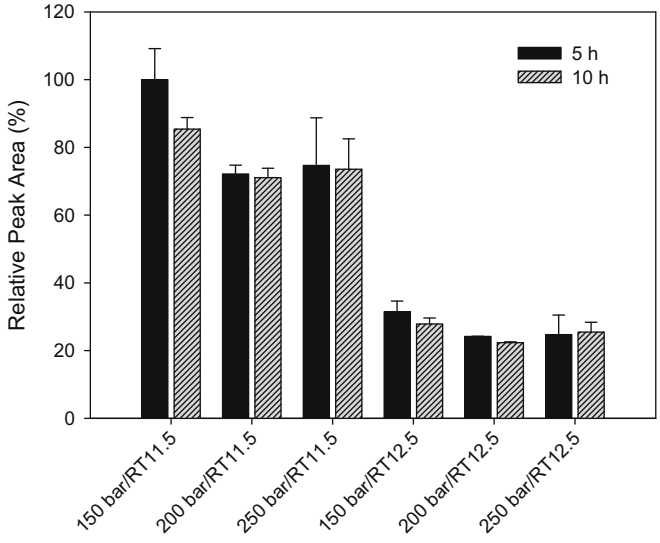

Fig. 3. Production of caffeic acid glucosides by SPase in SC-CO_2 at 42 °C and at 150, 200, or 250 bar. RT 11.5 and RT 12.5 represent the retention times of the peaks corresponding to caffeic acid diglucoside and monoglucoside, respectively.

dry gas was set at 10 l/min, and the nebulizer pressure was at 60 psi. In negative mode, the dry temperature was maintained at 350 °C, and the mass spectrometer was scanned from 50 to 600 m/z at 200 ms for maximum accumulation time, and 100,000 for smart target of ion charge control (ICC) in ultrascan mode. For MS/MS analysis, the mass spectrometer was scanned from 50 to 600 m/z.

3. Results and discussion

3.1. Transglucosylation of caffeic acid in aqueous buffer

Recombinant SPase, originating from *B. longum* in *E. coli*, was incubated with a mixture of sucrose and caffeic acid in potassium phosphate buffer (pH 6.8). In the transglucosylation reaction, sucrose and caffeic acid were utilised as a donor and an acceptor, respectively. In the LC/MS analysis, after the reactions were completed, we detected two major peaks other than that of caffeic acid (Fig. 1A); these peaks were considered to be the major transfer products from the transglucosylation of caffeic acid and sucrose. Also, the areas of these two major peaks increased with the reaction time in the aqueous medium with a significance level of 1% (Fig. 2). Importantly, these peaks appeared at retention times earlier than that of caffeic acid, thus indicating that increased aqueous solubility was conveyed by the glucosyl residue. In addition to these two major transfer products, we also detected three other peaks in the LC/MS analysis of the reaction mixture.

3.2. Transglucosylation of caffeic acid in aqueous-SC-CO_2 medium

Since polyphenols such as caffeic acid have low aqueous solubility, most previous studies on transglucosylations of polyphenols using SPase were carried out in a 2% (v/v) aqueous methanol solution (Kitao et al., 1993, 1995). In this study, we conducted the

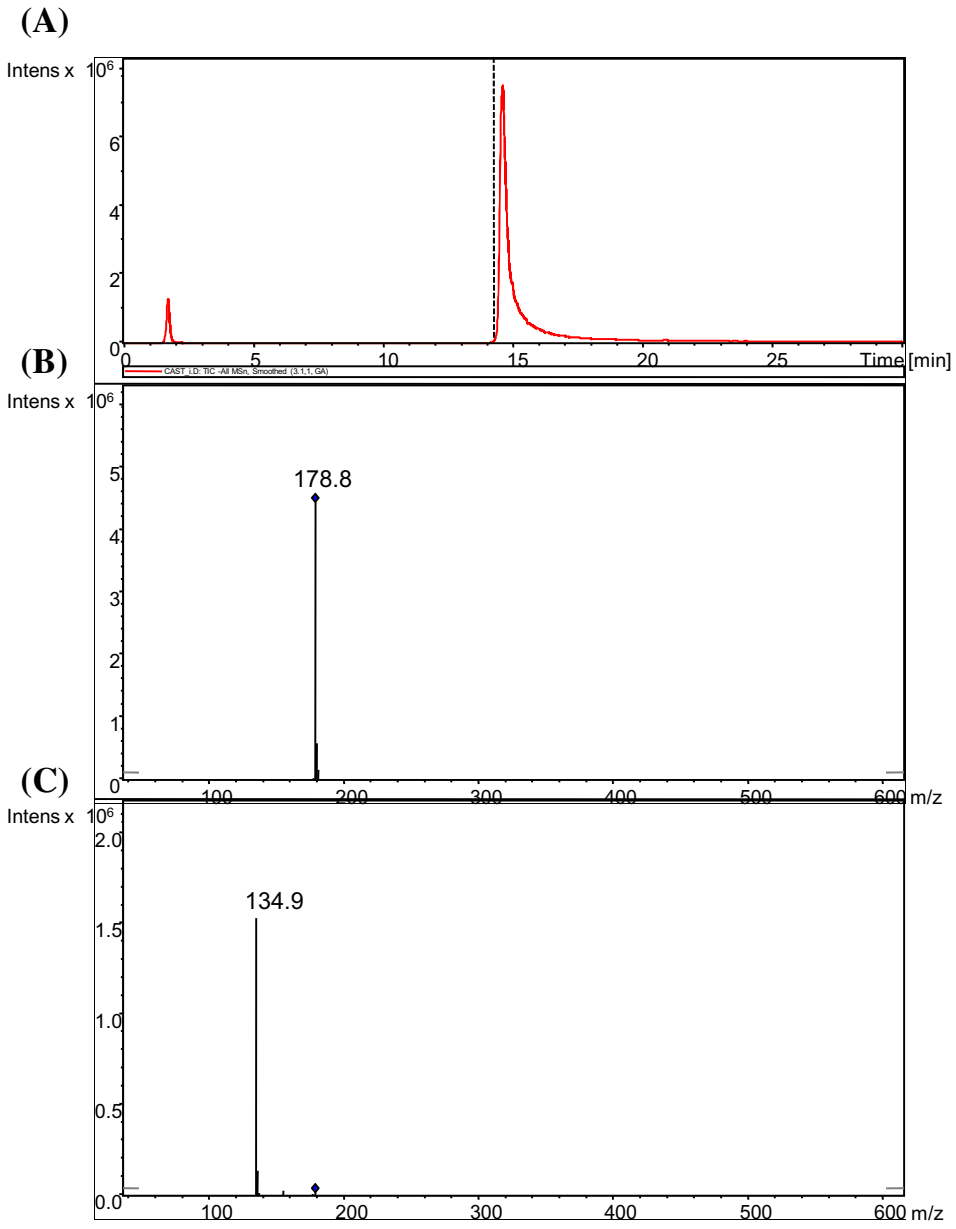

Fig. 4. The total ion chromatogram and the MS spectra of the authentic standard of caffeic acid (CA). (A) Total ion chromatogram of caffeic acid. (B) MS spectrum of caffeic acid. (C) MS/MS spectrum of caffeic acid. The numbers with arrows indicate the mass-to-charge ratio (*m/z*) of each peak.

transglucosylation of caffeic acid in SC-CO_2 under variable pressure at 42 °C for either 5 or 10 h. In the LC/MS analysis, we also detected the same peaks of two major transfer products, although the peak areas were smaller in the aqueous-SC-CO_2 medium than in the aqueous medium (Fig. 1B). These results imply that the reaction by SPase was not facilitated by SC-CO_2 but rather was repressed, possibly by the deactivation of SPase by SC-CO_2.

As shown in Fig. 3, the product yields from the reactions in the aqueous-SC-CO_2 medium at 200 and 250 bar were significantly lower than those at 150 bar. Also, although we increased the reaction time from 5 to 10 h, we did not see significant differences in the reaction product yields. For the product with a retention time of 11.5 min at 150 bar, its peak area rather decreased upon extending the reaction time from 5 to 10 h. The lower product yield in the aqueous-SC-CO_2 medium compared to the aqueous buffer and the low conversion at a higher pressure of SC-CO_2 could be attributed to the deactivation or denaturation of enzyme by SC-CO_2. In the binary system of water-SC-CO_2, pH of the heavy phase was calculated to be near 3.1 at 150–250 bar at 40 °C (Spilimbergo, Bertucco, Basso, & Bertoloni, 2005). When a phosphate-buffered saline was treated by SC-CO_2 and was taken out, the initial pH before SC-CO_2 treatment (pH 7.0) decreased to pH 5.5 (Kim, Rhee, Kim, Lee, & Kim, 2007). Such pH reduction of the aqueous phase is considered to be due to the generation of carbonic acid caused by the dissolution of CO_2 into the aqueous phase. Therefore, the reaction with SPase under SC-CO_2 probably suffered from either low enzyme activity or deactivation of the enzyme under a much more acidic condition than its initial pH 7.0. In the case of SPase, when pH was reduced from 7.0 to 6.0, its activity decreased by half (Kitao et al., 1993).

Despite the low yield in the aqueous-SC-CO_2, this is the first attempt to conduct an enzymatic synthesis of phenolic compounds in the SC-CO_2 phase. Further studies, beyond the scope of this work, could evaluate the polarity and solubility of caffeic acid and sucrose in SC-CO_2 to improve both the reaction yield and rate of polyphenol transglucosylation. In addition, such studies could effectively fine-tune important factors, such as pH of the reaction medium, reaction temperature and pressure, and the presence of co-solvents.

3.3. Mass fragmentation pattern of caffeic acid

To confirm that the transfer products tentatively observed by LC/MS in Fig. 1 were caffeic acid glucosides formed by the recombinant SPase, we carried out LC/MS/MS analysis. Since the molecular masses of both caffeic acid and glucose are 180, a caffeic acid

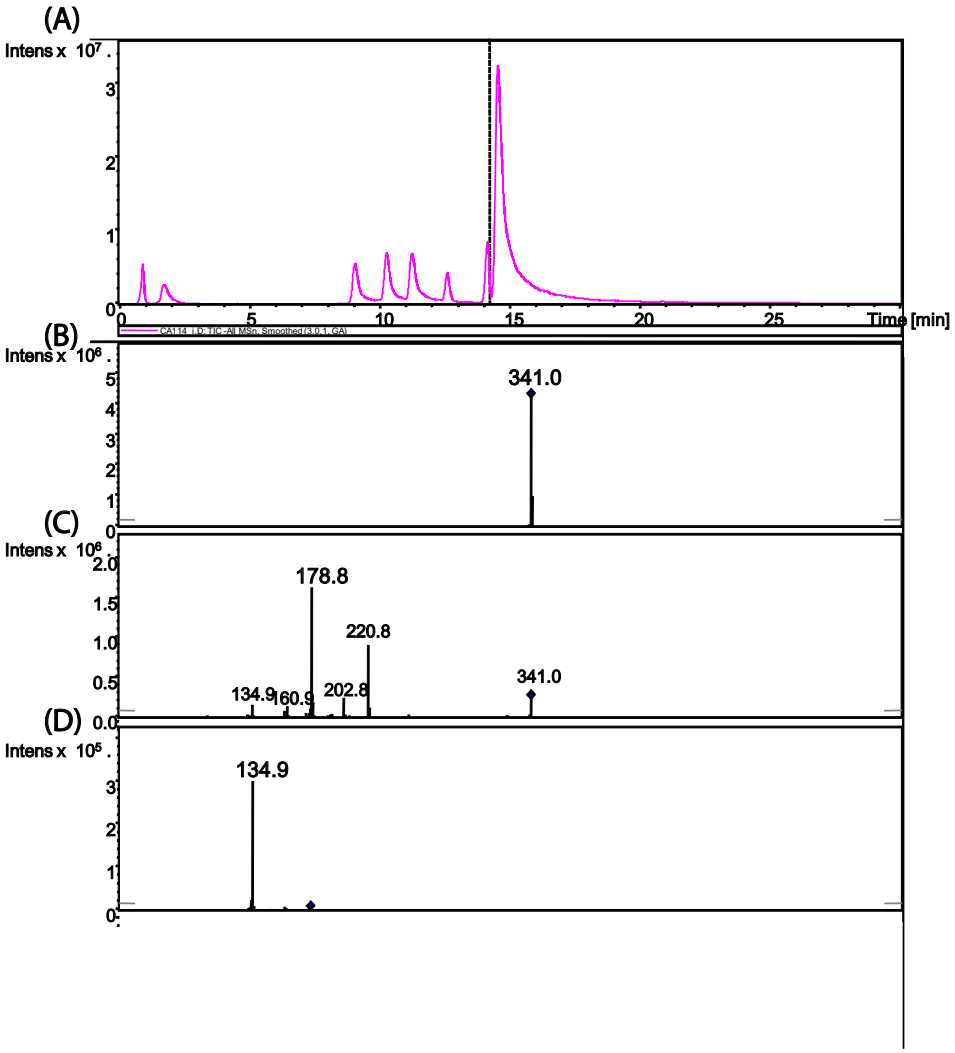

Fig. 5. The total ion chromatogram and the MS spectra of the reaction product of the recombinant SPase in aqueous medium. (A) Total ion chromatogram of reaction products. (B) MS spectrum of the peak with the 12.5 min retention time. (C) MS/MS spectrum of the peak with the 12.5 min retention time. (D) MS/MS/MS spectrum of the peak with the 12.5 min retention time. The numbers with arrows indicate *m/z* for each peak.

glucoside has a molecular mass of 342. Fig. 4 shows the mass spectrum of a dehydrogenated caffeic acid at 179 m/z, and further analysis by LC/MS/MS produced an ion at 135 m/z.

3.4. Analysis of enzymatic reaction products by LC/MS/MS

Fig. 5 shows the total ion chromatogram and the mass spectra of the reaction products produced by the recombinant SPase in aqueous medium. The major peak, observed at a retention time of 12.5 min in the total ion chromatogram, was at 341 m/z (Fig. 5B); further fragmentation of this molecular ion generated a molecular ion at 179 m/z in the MS/MS spectrum (Fig. 5C) and a molecular ion at 135 m/z in the MS/MS/MS spectrum (Fig. 5D). These analytical results are in accordance with the characterised *m/z* values for glucose and caffeic acid in the mass fragmentation pattern of glucose and caffeic acid, respectively, implying that the reaction product was a caffeic acid monoglucoside.

We also analysed the molecule assigned to the peak at a retention time of 11.5 min. Based on its molecular mass of 504, we proposed that it was a caffeic acid diglucoside consisting of one caffeic acid and two glucose molecules. We considered the other three products to be byproducts of the enzyme reaction, since they did not produce molecular ions with either 179 m/z or 135 m/z. These analytical results agree with those from the aqueous-SC-CO_2 medium.

Caffeic acid has three phenolic OH groups; hence, there are three regions to which glucose can attach. Fragmenting the caffeic acid by electrospray ionisation (ESI) produced a molecular ion of 135 m/z, as in Fig. 4C. In Fig. 6, panels A–C depict the structure of caffeic acid, the predicted structure of the fragmented molecular ion of caffeic acid, and the predicted structure of the caffeic acid glucoside, respectively. As shown in Fig. 5, we found that the compound with a 12.5 min retention time fragmented into two molecular ions with 179 and 221 m/z. Based on these fragmentation patterns, we presumed that glucose had attached to the A region of caffeic acid (see Fig. 6A). Studies to date have reported the enzymatic conversion of transglucosylation of caffeic acid to caffeic acid-3-*O*-glucoside or caffeic acid-4-*O*-glucoside, using UDP-glucose:glucosyltransferase or hydroquinone glucosylation enzyme (Lim, Higgins, Li, & Bowles, 2003; Nishimura, Kometani, Takii, Terada, & Okada, 1995), but there has been no report on such reactions with caffeic acid using SPase.

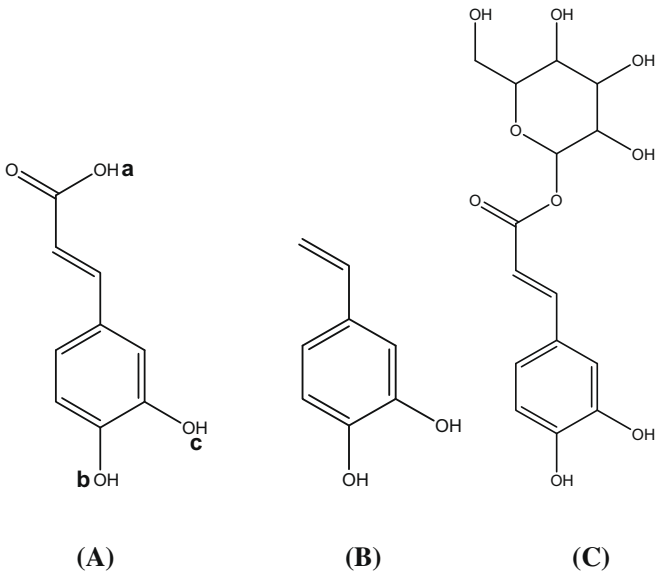

Fig. 6. Structures of (A) caffeic acid, (B) the predicted molecular ion with the 135 m/z of caffeic acid and (C) the predicted structure of the caffeic acid glucoside with a retention time of 12.5 min. Glucose could attach to either a, b, or c in the caffeic acid molecule shown in (A).

While many researchers have conducted transglucosylation of polyphenols, based on the structure of gallic acid and flavones, with SPase, this is the first to produce transglucosylated caffeic acids on the basis of cinnamic acid. Also, it is the first attempt to perform such an enzymatic reaction in an aqueous-SC-CO_2 medium. In a future study, we could improve the reaction yield and rate of polyphenol transglucosylation in SC-CO_2 by evaluating the polarity and solubility of substrates and tuning the important factors effectively.

4. Conclusions

A novel transglucosylation reaction from sucrose to caffeic acid by a recombinant SPase from *B. longum* was carried out in aqueous buffer and aqueous-SC-CO_2 media. By the enzymatic reactions, using the SPase with caffeic acid and sucrose, two transfer products were verified to be caffeic acid mono- and di-glucosides, using LC/MS/MS analysis.

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