Transglycosylation of Neohesperidin Dihydrochalcone by *Bacillus stearothermophilus* Maltogenic Amylase

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Neohesperidin dihydrochalcone (NHDC), a sweet compound derived from citrus fruits, was modified to a series of its oligosaccharides by transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase (BSMA). Maltotriose as a donor was reacted with NHDC as an acceptor to glycosylate for the purpose of increasing the solubility of NHDC. Maltosyl-NHDC was a major transglycosylation product among the several transfer products by TLC analysis. The structure of the major transglycosylation product was determined to be maltosyl-α-(1,6)-neohesperidin dihydrochalcone by MALDI-TOF/MS and ^{1}H and ^{13}C NMR. Maltosyl-NHDC was 700 times more soluble in water and 7 times less sweet than NHDC.

Keywords: *Bacillus stearothermophilus maltogenic amylase (BSMA); transglycosylation; neohesperidin dihydrochalcone (NHDC); maltosyl-α-(1,6)-neohesperidin dihydrochalcone*

INTRODUCTION

Two groups of flavonoid glycosides contained in citrus fruits are mainly found in their peels. They include the tasteless rutinosyl glycosides and the extremely bitter neohesperidosyl derivatives (Sachiko et al., 1994). Due to the unacceptable bitter taste of the flavonoid glycosides, efforts to reduce the bitter taste by modifying the structure have been attempted during the past few decades (Puri et al., 1996).

Horowitz and Gentili (1969) succeeded in the conversion of the bitter neohesperidosyl glycoside into intensely sweet flavonoid derivatives. One of the converted flavanone glycosides, neohesperidin dihydrochalcone (NHDC), was 1000−1500 times sweeter than sucrose and 2 times sweeter than saccharin (Whitelaw et al., 1991). NHDC could be considered as a potential substance in the food industry not only as a sugar substitute but also as a flavor enhancer (Inglett et al., 1969). However, the solubility of NHDC is very low, and its application is quite limited.

The transglycosylation reaction has been used to improve certain characteristics of food materials such as rutin, stevioside, and rubusoside by increasing the solubility or reducing the bitter taste (Kometani et al., 1996). Recently, a new type of amylase, named *Bacillus stearothermophilus* maltogenic amylase (BSMA), was screened from *B. stearothermophilus* by our group; it not only has hydrolytic activity but also has transglycosylation activity (Cha et al., 1998). The maltogenic amylase can transfer the mono- or disaccharide to the target molecule by forming either α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkages. The mechanism of transglycosylation was first illustrated by determination of crystal structure of the enzyme (Kim et al., 1999)

In this study, NHDC was modified using the transglycosylation activity of BSMA to improve its solubility. The transfer products were analyzed by TLC and purified using gel permeation chromatography. Also the structure of the major transglycosylated product was determined by MALDI-TOF/MS and ^{1}H and ^{13}C NMR. Finally, the solubility and the sweetness of the transglycosylated NHDC were examined.

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MATERIALS AND METHODS

Materials. BSMA was obtained from recombinant *E. coli* DH5α that harbors plasmid pSG18, which originated from *B. stearothermophilus* ET1 (Cha et al., 1998). NHDC (neohesperidin dihydrochalcone) was purchased from Sigma Chemical Co. (St. Louis, MO).

Enzyme Assay. The activity of BSMA was assayed using 1% β-CD (β-cyclodextrin) in 50 mM Na-citrate buffer (pH 6.0). BSMA (100 μL) diluted in the same buffer were reacted with 500 μL of 1% β-CD and 400 μL of the buffer at 55 °C for 30 min. The reaction was stopped by adding 3 mL of DNS solution (3,5-dinitrosalicylic acid 10.6 g, NaOH 19.8 g, sodium potassium tartrate 306 g, phenol 7.6 mL, sodium metabisulfite 8.3 g, and water 1416 mL) and colorized by heating at 100 °C for 5 min. The absorbance of the mixture was measured by a spectrophotometer (Ultrospec III, Pharmacia LKB, Uppsala, Sweden) at 575 nm. One unit of enzyme activity was defined as the amount of enzyme producing the reducing sugar equivalent to one unit change of absorbance at 575 nm.

Transglycosylation to NHDC by BSMA. The reaction mixture for the transglycosylation consisted of 5% NHDC (500 mg) as an acceptor, 5% maltotriose (500 mg) as a donor, and 10 units of BSMA/mg of maltotriose in 50 mM Na-citrate buffer (pH 6.0). After reacting at 55 °C for 24 h, it was boiled for 5 min to stop the reaction and centrifuged at 10 000 rpm for 10 min to remove the insoluble NHDC. The reaction products were analyzed by TLC as follows. The TLC analysis was performed using a K5F silica gel 150A TLC plate (Whatman, U.K.) and a solvent system of chloroform/methanol/water (v/v/v = 65:35:10). The developed plate was dried and visualized under a UV detector (CAMAG Reprostar 3, Muttenz, Switzerland) at 254 nm. The plate was sprayed with 0.3% (w/v) *N*-1-naphthylethylenediamine, 5% (v/v) H_2SO_4 in methanol and heated at 110 °C for 5 min.

Purification of the Major Transfer Products. The reaction mixture was concentrated by Speed Vac SC-110

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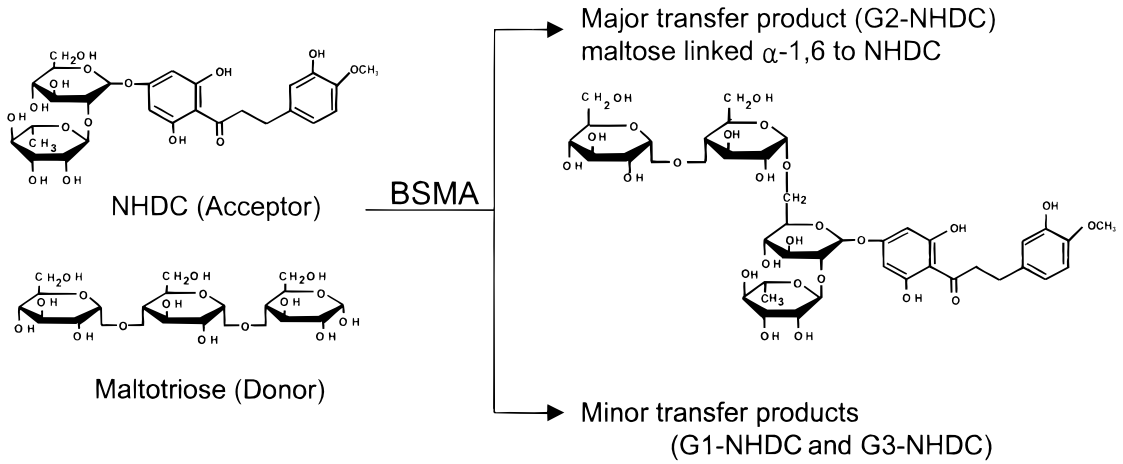

Figure 1. Transglycosylation reaction of NHDC by BSMA (neohesperidin dihydrochalcone, NHDC; glucose, G1; maltose, G2; maltotriose, G3).

(Savant Instruments, Inc., Holbrook, NY), loaded onto the Sephadex LH-20 column, and eluted with water and 50% ethanol at a flow rate of 1 mL/min at room temperature. The fractions containing the transfer products were confirmed by TLC. The major transfer product was separated by preparative TLC. The amount of maltosyl-NHDC was estimated by a UV spectrophotometer at 282 nm.

Determination of the Structure for the Transfer Product. *MALDI-TOF/MS Analysis.* Matrix-assisted laser desorption ionization with time-of-flight (MALDI-TOF) mass spectrometry was used to measure the molecular weight of the major transfer product. Voyager-DE (PerSeptive Biosystem, Framingham) system was adopted using α-cyano-4-hydroxycinnamic acid (α-CHCA) as a matrix and des-Arg^1-bradykinin for calibration. The purified sample (1 μL) with the same volume of α-CHCA was placed into the sample well and allowed to dry. The sample plate was then located into the Voyager-DE biospectrometry workstation. Workstations were operated with an accelerating voltage of 20 kV. The molecular ion peaks were expected to appear as sodium or potassium adducts due to the reaction solution which including sodium potassium tartrate or sodium citrate.

^{1}H and ^{13}C NMR Analyses. The ^{1}H and ^{13}C NMR spectra of the major transfer product were measured in DMSO-d_6 with tetramethylsilane (TMS) as the internal reference and recorded with a Varian Inova-300 (Palo Alto, CA) at 26.5 °C.

Measurement of Solubility. The solubility and the sweetness of the transfer product, maltosyl-α-(1,6)-NHDC, were evaluated by comparing with those of NHDC (Shin et al., 1995). NHDC and maltosyl-NHDC (60 mg) were suspended in distilled water (50 μL) by vortexing at 25 °C, respectively. After removing the insoluble components by centrifugation at 10 000 rpm for 10 min, the amount of sample in the supernatant was measured using a UV spectrophotometer at 282 nm.

Sensory Evaluation. Each threshold value of NHDC and maltosyl-NHDC was measured by the method of Patton and Josephson (1957). The samples were prepared containing the compound in the range from zero concentration to well above the anticipated threshold area (0.001−0.5 mM). The tests were continued until the six panelists could detect the taste of the sample.

Statistical Analyses. Statistical analyses for solubility and sweetness of NHDC and maltosyl-NHDC were performed by analysis of variance and pairwise comparisons (Bonferroni's *t*-test) using SigmaStat for Windows, version 1.0 (Jandel Corp., San Rafael, CA).

RESULTS AND DISCUSSION

Transglycosylation of NHDC by BSMA. NHDC was transglycosylated by BSMA, which has hydrolysis and transglycosylation activities (Cha et al., 1998). The BSMA transglycosylation reaction of NHDC is shown in Figure 1. The transfer products were purified using Sephadex LH-20 column chromatography (Figure 2, lane 1). The major transfer product was purified by preparative TLC as shown in Figure 2.

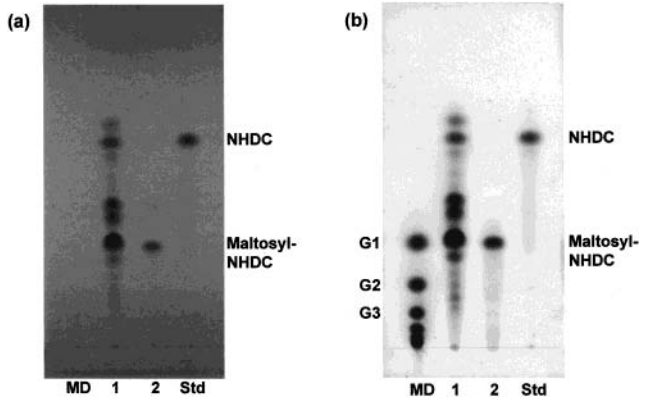

Figure 2. TLC analysis of transfer products by (a) UV at 254 nm and (b) sulfuric acid treatment: lane MD, maltodextrin standards of glucose (G1), maltose (G2), and maltotriose (G3); lane 1, transfer products after Sephadex LH-20 column chromatography; lane 2, purified maltosyl-NHDC; lane Std, NHDC.

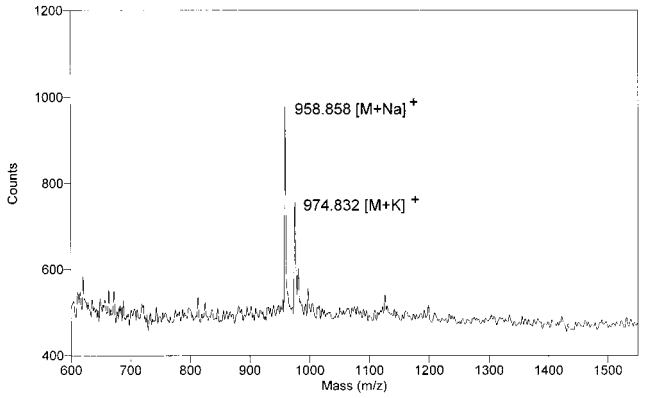

Figure 3. Molecular weight of the purified maltosyl-NHDC using MALDI-TOF/MS.

Structure of the Transfer Product. The molecular weight of the purified transfer product was determined by MALDI-TOF/MS as shown in Figure 3. It was estimated to be 936 Da from the two molecular ion peaks appearing at *m/z* 958.858 ($[M + Na]^+$) and 974.832 ($[M + K]^+$). The values were consistent with the calculated molecular masses of maltosyl-NHDC sodium and potassium adducts, respectively. The results

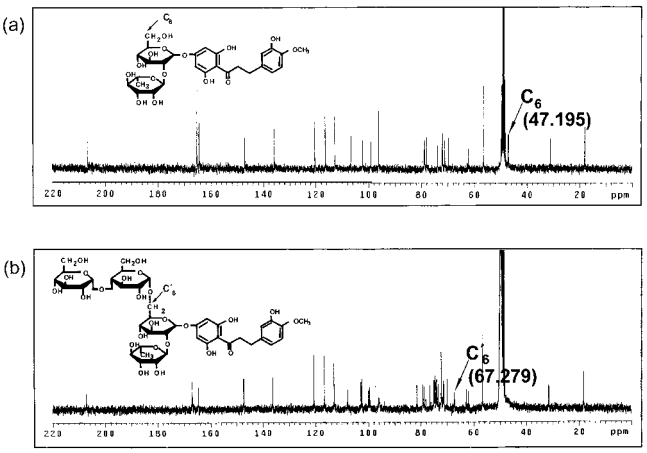

Figure 4. ^{13}C NMR spectra of (a) NHDC and (b) maltosyl-NHDC.

Table 1. Solubility and Sweetness Threshold Value of NHDC and Maltosyl-α-(1,6)-NHDC

compd	solubility (mM, 25 °C)	relative solubility	threshold value (mM)	relative sweetness
NHDC	1.45 (±0.061)	1	2.24×10^{-3} ($\pm 2.00 \times 10^{-4}$)	100
maltosyl-α-(1,6)-NHDC**	1010.67 (±24.23)	699.2 (±13.90)	1.60×10^{-2} ($\pm 1.00 \times 10^{-3}$)	14.0 (±0.35)

**Significantly different ($p < 0.01$).

implied that the major transfer product by BSMA was maltosyl-NHDC.

The ^1H and ^{13}C NMR analyses were carried out to configure the glycosidic linkage between maltose and NHDC (Caccia et al., 1996). Chemical shifts in the ^{13}C NMR spectrum of maltosyl-NHDC were compared with those of authentic NHDC. As shown in Figure 4, a large chemical shift was observed in maltosyl-NHDC which occurred at C-6 in the glucose moiety of NHDC, from 47.195 to 67.279 ppm. The additional carbon signals which seemed to be the transferred maltose were also found. Those results suggest that the transferred maltosyl group was attached to C-6 in the glucose moiety of NHDC and is therefore described as maltosyl-α-(1,6)-NHDC.

The results confirmed that the type of the glycosidic linkage and the transferred products were mainly derived from the specificity of the amylases. Kometani et al. (1996) reported that the transfer products were solely monoglucoside derivatives in all cases when they modified hesperidin, neohesperidin, and naringin with soluble starch by CGTase from *Bacillus* species. Also the type of the glycosidic linkage such as α-(1,3) or α-(1,4) depended on the structure of the acceptor molecule.

Solubility and Sensory Property. As shown in Table 1, maltosyl-α-(1,6)-NHDC could be dissolved in water up to 945 mg/mL (1010 mM), while NHDC was soluble only up to 0.89 mg/mL (1.45 mM). The solubility of the transfer product, maltosyl-α-(1,6)-NHDC, was approximately 700 times greater than that of NHDC. The tendency was consistent with the previous results from other flavonoid glycosides (Lee et al., 1999; Kometani et al., 1996).

The intensity of sweetness of maltosyl-α-(1,6)-NHDC was measured by comparison with that of NHDC. Table 1 shows that the threshold sweetness value of the transfer product was 1.6×10^{-2} mM, while that of NHDC was 2.24×10^{-3} mM. Therefore, the major transfer product, maltosyl-α-(1,6)-NHDC, was about 7 times less sweet than NHDC.

These findings indicate that the solubility of the potent sweetener NHDC was increased by transglycosylation of NHDC using BSMA. This result also implies that the maltogenic amylase containing the transglycosylation activity could be widely used to improve the characteristics of the food components.

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