Immobilization of Thermostable Maltogenic Amylase from *Bacillus stearothermophilus* for Continuous Production of Branched Oligosaccharides

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A new thermostable maltogenic amylase isolated from *Bacillus stearothermophilus* (BSMA) was immobilized and characterized for continuous production of branched oligosaccharides (BOS). Immobilized BSMA had characteristics similar to those of the free form, except its stability with respect to temperature and metal ions was improved. The optimum condition for continuous production of BOS using a packed bed column reactor of immobilized BSMA was a flow rate of 11 mL/h and a 20% substrate concentration. As the flow rate or concentration of the substrate increased, generally, the content of sugars having a higher molecular weight were increased and vice versa, suggesting the possibility of controlling the composition of BOS by adjusting the above two factors. The yield of BOS was maintained at 50% up to 20 days and gradually decreased to 40% over 37 days of continuous process. About 93% high-purity BOS was obtained by removing glucose and maltose by yeast fermentation.

Keywords: *Branched oligosaccharides; maltogenic amylase; Bacillus stearothermophilus; immobilization; continuous production*

INTRODUCTION

As one of the functional foods, branched oligosaccharides have several favorable properties. They are being used as a substitute for sucrose due to their low energy (2 kcal/g) and low cariogenic properties and are known to improve the intestinal microflora by stimulating the growth of *Bifidobacteria* and suppressing the multiplication of harmful bacteria (Cordt et al., 1992; Park, 1992; Tomomatsu, 1994). In addition, the physical properties, including high hygroscopicity, low viscosity, and a high freezing point, are the merits of branched oligosaccharides for their wide application in food products. For example, the product containing branched oligosaccharides has a lower water activity than other sugars and consequently has a longer shelf life by suppressing the growth of microorganisms (Cheong et al., 1996).

Recently, a new thermostable maltogenic amylase from *Bacillus stearothermophilus* (BSMA) has been isolated and characterized in this laboratory. The molecular mass and optimum temperature of BSMA were 62 kDa and 55 °C, respectively. BSMA not only hydrolyzes starch molecules but also transfers small units of sugar and forms α-1,6 linkages. When BSMA was incubated with 30% liquefied corn starch, it mainly produced branched tetraose, panose, and isomaltose, accounting for 66% of the total sugar. Therefore, BSMA appears to be highly suited for the production of branched oligosaccharides.

For mass production of branched oligosaccharides, the enzyme must be immobilized for the continuous process. Additional advantages of using immobilized enzymes include the possibility of reusing the enzyme, the minimal contamination of food product with added enzymes, the facility in enzymatic reaction control, etc. (Hultin, 1983).

The purposes of this study were to characterize immobilized BSMA and to develop a continuous production system for high-purity branched oligosaccharides by using BSMA.

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MATERIALS AND METHODS

Bacterial Culture. A gene encoding maltogenic amylase from *B. stearothermophilus* (BSMA) was cloned and expressed in *Escherichia coli* to increase the production of the enzyme (Cha et al., 1997). In brief, chromosomal DNA of *B. stearothermophilus* was partially cleaved by *Hin*dIII and ligated to the same restriction site of pUC18. A genomic DNA library was constructed by transforming the ligation mixture into *E. coli*, and the resulting transformants were screened for starch hydrolysis activity. The *E. coli* transformant was precultured in 50 mL of LB (Luria-Bertani) broth containing 50 mg/L ampicillin for 12 h at 37 °C, transferred to 3 L of the same broth, and cultured for 12 h under the same conditions, using a stirred tank fermenter (Korea Fermentor Co., Inchon, Korea) with aeration.

Purification of the Enzyme. The cells were collected by centrifugation at 6000*g* for 10 min, resuspended in 50 mM Tris buffer (pH 7.5), sonicated for 5 min in an ice bath, and centrifuged again to obtain the supernatant. It was fractionated with 20−50% ammonium sulfate, and the precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer for 8 h at 4 °C. The crude enzyme was purified further by using DEAE-TOYOPEARL 650 (Tosoh Co., Tokyo, Japan) and Mono Q HR 5/5 columns (Pharmacia, Uppsala, Sweden) connected to a fast protein liquid chromatography (FPLC) system (Pharmacia).

Immobilization of BSMA. The method described by Cordt et al. (1992) was followed with minor modification. Six grams of CPC-silica carrier (Fluka, Buchs, Switzerland) and 20 mL of 2.5% glutaraldehyde in distilled water (Sigma, St. Louis, MO) were mixed and incubated at 27 °C for 2 h with gentle shaking. The carrier was washed with distilled water several

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times to remove residual glutaraldehyde and suspended in 50 mM phosphate buffer (pH 6.0). Then, 6 mL of purified BSMA (4000 CU/mL) was added to the buffer and incubated in a shaking incubator for 12 h at 27 °C, where 1 unit of β-cyclodextrin- hydrolyzing activity (CU) was defined as the amount of enzyme producing reducing sugar equivalent to 1 unit of change of absorbance at 575 nm. After immobilization, the beads were washed several times with phosphate buffer (pH 7.0) before use.

Measurement of Enzyme Activity. The activity of free BSMA was measured by reacting with a 0.5% β-cyclodextrin (CD) solution for 30 min at 55 °C and measuring the difference in absorbance by the DNS method (Miller, 1959).

The activity of immobilized BSMA was measured as follows. Two and one-half milliliters of 50 mM citrate buffer (pH 6.0) and 2.5 mL of substrate solution (1% β-CD) were mixed in a beaker and prewarmed to 55 °C. Then, a specific amount of immobilized BSMA was added and reacted for 10 min by shaking at 140 strokes/min. The absorbance of the solution was measured as described above using the DNS method, and the used beads were dried to obtain the dried weight value. The activity of immobilized enzyme was calculated by the equation given below:

$$\mathrm{CU} = \frac{\Delta\ \text{Abs at 575 nm}}{\text{unit weight (g) of immobilized BSMA}} \quad (1)$$

Optimum pH and pH Stability of Immobilized BSMA. The optimum pH and pH stability of immobilized BSMA were measured as follows. One-fourth of a milliliter of universal buffer solution at each pH (4.0−10.0) containing 0.5% β-CD was prewarmed to 55 °C, to which added was 10 mg (wet weight) of immobilized BSMA suspended in universal buffer at the same pH, and reacted for 10 min. To check the pH stability, 10 mg of immobilized BSMA was sustained in universal buffer at each pH for 30 min at 55 °C, and reacted with 0.5% β-CD at pH 8.0 for 10 min. The enzyme activity was measured as described above.

Optimum Temperature and Thermostability of Immobilized BSMA. The optimum temperature for immobilized BSMA was determined as follows. One-fourth of a milliliter of 50 mM citrate buffer (pH 6.0) containing 1% β-CD and 0.2 mL of the same buffer were mixed and prewarmed to 45, 50, 55, 57, 60, 65, or 70 °C for 5 min. Then, 10 mg (wet weight) of immobilized BSMA was added to each tube and reacted for 30 min at each temperature, and the activity of the enzyme was measured as described above. To determine the thermal stability, 0.5 g (wet weight) of immobilized BSMA was kept at 55, 60, 65, or 70 °C for various times (5, 10, 15, 20, 25, 30, and 35 min) and the residual activity was measured as described above.

Metal Ions' Effect on the Activity of BSMA. To see the effect of metal/ion species on the activity of BSMA, immobilized BSMA was reacted with 0.5% β-CD in 5 mM metal/ion solutions of $MgCl_2$, $FeCl_3$, $NiCl_3$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, LiCl, $MnCl_2$, $Na_2S_2O_3$, $BaCl_2$, $CoCl_2$, and $AgNO_3$ and the activity measured as described above.

Continuous Production of Branched Oligosaccharides (BOS). Branched oligosaccharides were produced by using a packed bed column reactor. Six grams of immobilized BSMA was packed in a column (10 mm × 150 mm, Amicon Co.) with a water jacket, and liquefied corn starch solution (Low DE, Samyang Co., Seoul, Korea) was added as a substrate. To optimize the production of BOS, the continuous process was run at various flow rates (1−20 mL/h) and substrate concentrations [5−30% (w/v)]. The column temperature was maintained at 50 °C using a circulator, and the flow rate of the substrate was kept constant by using a peristaltic pump.

Production of High-Purity BOS. The BOS mixture was fermented with immobilized *Saccharomyces cerevisiae* var. *ellipsoideus* cells to remove glucose and maltose from the BOS mixture according to Yoo et al. (1995). *S. cerevisiae* var. *ellipsoideus* (IFO 1950) obtained from the Korean Culture Center of Microorganisms was cultured in YPD (yeast extract/bactopeptone/dextrose) broth for 18 h at 27 °C with agitation.

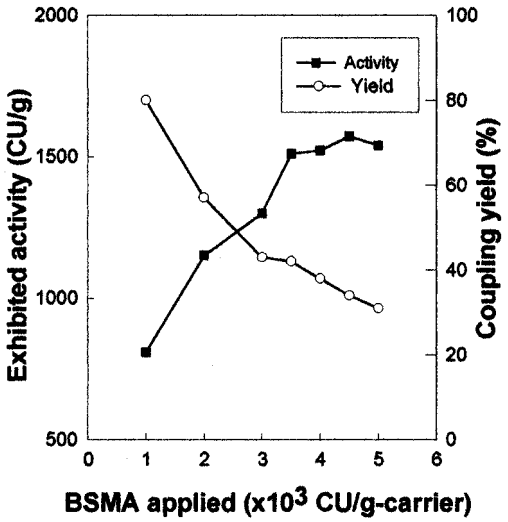

Figure 1. Relationship between the amount of BSMA applied and the exhibited activity of immobilized BSMA.

The collected cells were added to the carrier, granular cellulose, suspended in distilled water which had been hydrated with water and dried for 30 min at 90 °C, and the mixture was incubated for 15 h at 10 °C with intermittent agitation. The immobilized yeast cells were washed with distilled water three or four times before use.

Five grams of immobilized yeast cells and 10 mL of BOS were mixed well in a 100 mL flask, and the inner atmosphere was made anaerobic by connecting the flask with a fermentation tube. They were fermented for 2 days at 27 °C. After fermentation, high-purity BOS was obtained by sequential processes of ethanol evaporation with rotary vacuum evaporator and deodorization and decolorization by activated charcoal and ion exchange glass beads.

Analysis of BOS by High-Performance Ion Chromatography (HPIC). HPIC was carried out to analyze the sugar composition of BOS produced by using a CarboPac PA1 column (Dionex, Sunnyvale, CA) and a pulsed amperometric detector (PAD, Dionex). All solvents were degassed by vacuum stirring and filtered through a poly(vinylidene difluoride) membrane with a 0.45 μm pore size. Samples were eluted at 1.0 mL/min with a linear gradient of 150 mM NaOH (100 to 70%) and 150 mM NaOH containing 600 mM sodium acetate (0 to 30%) for 30 min. Twenty microliters of a 0.02% sample solution was injected into the column.

RESULTS AND DISCUSSION

Purification and Immobilization of BSMA. After sequential purification from the cell extract, BSMA having a specific activity of 1.59×10^3 CU/mg was obtained and used for immobilization. The yield and purification of BSMA at this point were 3.65% and 6.95-fold, respectively.

As the amount of BSMA added per gram of carrier increased, the activity of immobilized enzyme increased and reached an equilibrium when 3500 CU/g carrier was added. The coupling yield, however, was inversely proportional to the amount of BSMA added (Figure 1), and the immobilization yield at the equilibrium point was 43%. When the amount of applied enzyme was lower than 2000 CU/g, the coupling rate was much greater; however, the activity of enzyme per gram of the carrier was significantly reduced due to the fact that the absolute amount of the enzyme immobilized was reduced.

Characteristics of Immobilized BSMA. The characteristics of immobilized BSMA were similar to those of free BSMA. The optimum pH of immobilized BSMA

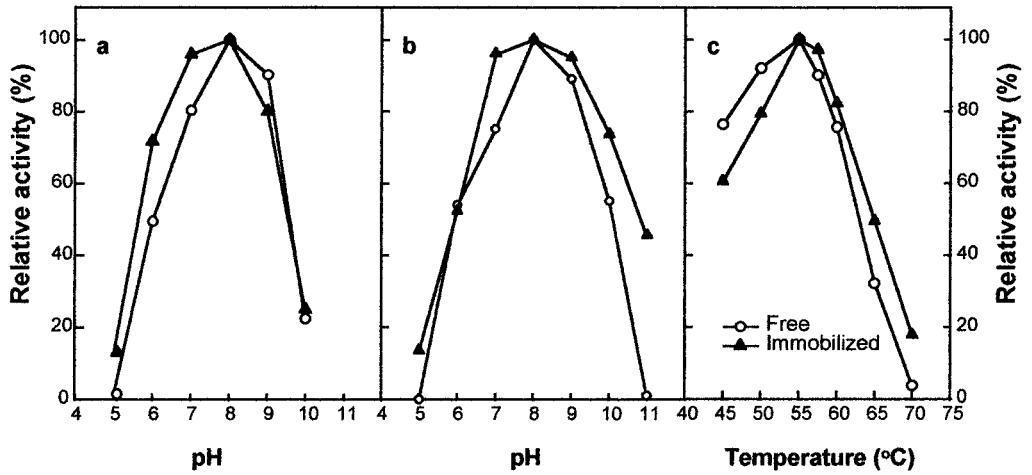

Figure 2. Characteristics of BSMA: (a) activity of BSMA at various pHs, (b) stability of BSMA at various pHs, and (c) activity of BSMA at various temperatures.

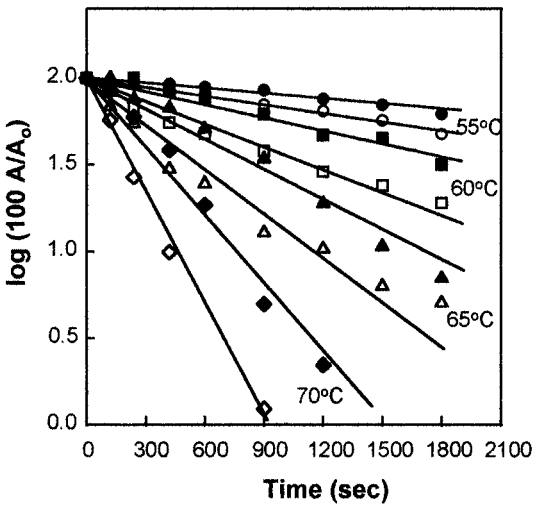

Figure 3. Stability of BSMA measured at various temperatures. Closed and open symbols are for the immobilized and free BSMA, respectively, at each temperature.

was around pH 7−8 (Figure 2a), and the overall pH profile was shifted toward lower pHs by about 1 unit. Immobilized BSMA was stable in the range of pH 7−9 (Figure 2b), and generally high relative activity and stability were retained in the wide range of pHs close to the optimum, which suggested that immobilization increased the stability of BSMA. The optimum temperature of BSMA at its free form, 55 °C, was still retained in immobilized enzymes; however, the relative activity at each temperature was generally increased by immobilization (Figure 2c). The thermostability of BSMA was significantly increased by immobilization at all temperatures tested (55−70 °C) (Figure 3). Consequently, *D* values of immobilized BSMA, which was defined as the heating time required to reduce the original activity of the enzyme by one log at a specific temperature, were increased about 1.6-fold at all temperatures tested (Table 1).

The activity of free BSMA was generally decreased by the presence of various metal/ion species in the solution; however, immobilized BSMA was affected less (Table 2). The relative activities of immobilized BSMA were 4.4-, 1.7-, 22.0-, 2.7-, 6.2-, and 1.6-fold greater than those of free forms in the presence of 5 mM $FeCl_3$, $ZnCl_2$, $CuCl_2$, $MnCl_2$, $Na_2S_2O_3$, and $CoCl_2$, respectively. The above results suggest that immobilization increased the stability of BSMA against various metal/ion species.

Table 1. Thermal Inactivation Kinetics of Free and Immobilized BSMA

	D values (s)[a]			
BSMA type	55 °C	60 °C	65 °C	70 °C
free	6000	2292	1026	456
immobilized	9996	3564	1644	678

[a] The heating time required to reduce the original activity of the enzyme by one log at a specific temperature.

Table 2. Effect of Various Metal/Ion Species on the Relative Activity of BSMA

	relative activity (%)[a]	
metal/ion (5 mM)	free	immobilized
none	100	100
$MgCl_2$	97.6	81.3
$FeCl_3$	16.1	72.1
$NiCl_2$	63.4	89.9
$ZnCl_2$	40.1	67.8
$CuCl_2$	0.2	4.4
$CaCl_2$	70.9	84.6
LiCl	95.4	90.8
$MnCl_2$	32.2	85.5
$S_2O_3^{2-}$	5.7	35.6
$BaCl_2$	104.3	99.2
$CoCl_2$	74.6	117.8
$AgNO_3$	0	0

[a] The percent activity of an enzyme compared with the activity without the presence of metal ions.

Continuous Production of Branched Oligosaccharides. The composition of BOS produced differed depending upon the type of process, the flow rate of the substrate, and the substrate concentration. The yield of BOS from the continuous process by immobilized BSMA was 50.8% (Table 3). Compared with the batch type process, the continuous process produced more glucose and maltose, and the overall content of BOS was decreased slightly in the continuous process. The results indicated that the composition of BOS was determined by the coupled reaction of the relative rate of hydrolysis, transglycosylation of BSMA, and the reaction time. BSMA is believed to hydrolyze maltooligosaccharides having a higher degree of polymerization (DP) first and second to transfer glucose and maltose units produced at the first stage to other units to form α-1,6 linkages. Therefore, BOS having a higher DP, including branched tetraose and pentaose, would be produced at the early stage of reaction, and as the reaction proceeded, they would be hydrolyzed to smaller oligosaccharides such as isomaltose, panose, and isopa-

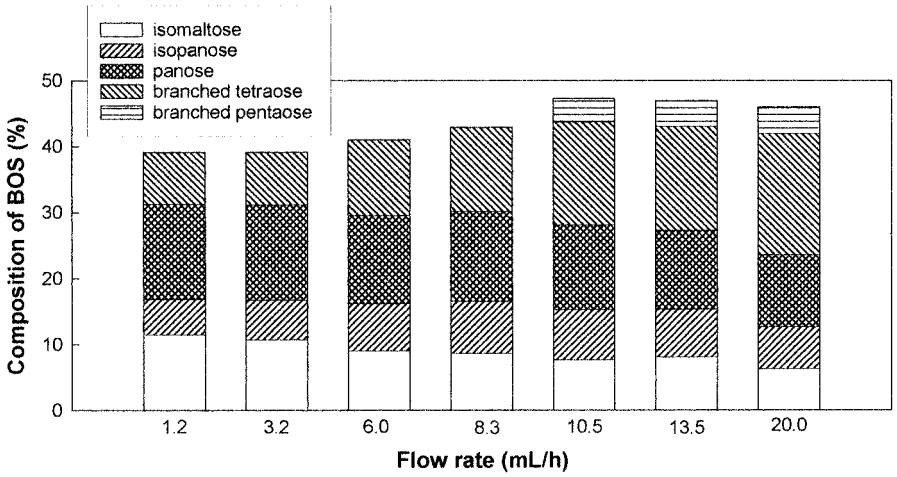

Figure 4. Continuous production of BOS at various flow rates of the substrate. The substrate was 20% liquefied corn starch.

Table 3. Composition and Total Amount of Branched Oligosaccharide Produced by Free and Immobilized BSMA[a]

enzyme and reaction type	composition of oligosaccharide (%)									total BOS (g/L)
	glucose	maltose	maltotriose	isomaltose	isopanose	panose	BOS-4	BOS-5	total BOS	
free BSMA	23.6	16.3	1.6	8.2	6.3	21.5	18.0	4.5	58.5	117.0
immobilized BSMA										
batch type	25.3	16.2	2.1	7.9	5.9	18.7	19.6	4.3	56.4	112.8
continuous type	28.4	17.4	3.4	8.7	6.4	13.0	17.9	4.8	50.8	101.6
continuous with yeast fermentation	–	1.4	5.9	10.5	14.3	22.6	30.8	14.5	92.7	101.6

[a] The substrate concentration was 20% (w/w).

nose, and the content of glucose and maltose would be increased as well. The decreased flexiblity of the enzyme due to immobilization might have allowed limited access to the acceptor oligosaccharides for glucose and maltose. Consequently, the hydrolyzing reaction would predominate transferring activity, which would partly explain the decreased content of total BOS in immobilized BSMA.

There was a change in the production and composition of BOS due to the flow rate in the continuous process (Figure 4). As the flow rate increased, the content of total BOS was increased gradually, reached the maximum at 10.5 mL/h, and then decreased slowly. The contents of branched tetraose and pentaose were increased; however, those of panose and isomaltose were decreased as well. That is, as the flow rate increased, the reaction time with the substrate was decreased and consequently the content of high-molecular mass oligosaccharide was increased, and vice versa. This result suggests that the composition of BOS can be adjusted by changing the flow rate of the substrate.

As the concentration of the substrate increased, the content of total BOS was increased rapidly at the low concentration and reached an equilibrium at a concentration of greater than 20% (Figure 5). Except panose which was maintained constant regardless of the substrate concentration, the contents of high-molecular mass sugars like branched tetraose and pentaose were increased and low-molecular mass sugars, including isomaltose, were decreased as the concentration of substrate increased. That is, as the concentration of substrate increased, BOS with a higher degree of polymerization were produced.

The optimum condition for maximum production of BOS with the continuous process was chosen to be 20% of the substrate concentration and a flow rate of 11 mL/h, and the effect of the operation time under the above conditions was observed in terms of the content of total BOS and sugar composition. There was little change in the composition of BOS, and the total content of BOS

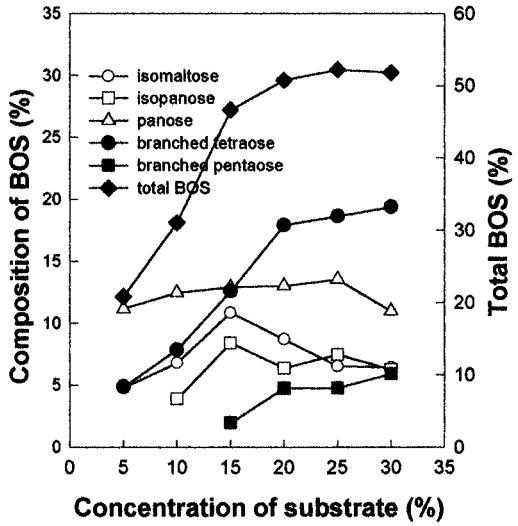

Figure 5. Continuous production of BOS at various substrate concentrations. The substrate was liquefied corn starch, and the flow rate of the substrate was 11 mL/h.

was maintained at about 50% up to 480 h (20 days) of operation time. This continuous process could be run up to 32 days; however, at 37 days of the continuous process, the yield of BOS was decreased to 80% (data not shown) probably due to partial inactivation of the immobilized enzyme. If the flow rate is made low at this stage, constant production of BOS with a similar composition of sugar should be possible by increasing the reaction time of the enzyme with substrate.

Production of High-Purity BOS. When the BOS solution produced with the continuous process was fermented by immobilized yeast (Figure 6), glucose disappeared completely and only the trace of maltose was observed. There was no change in the content of other nonfermentable sugars. By successfully removing glucose and maltose which comprised over 40% of BOS, we obtained 93% pure BOS (Table 3).

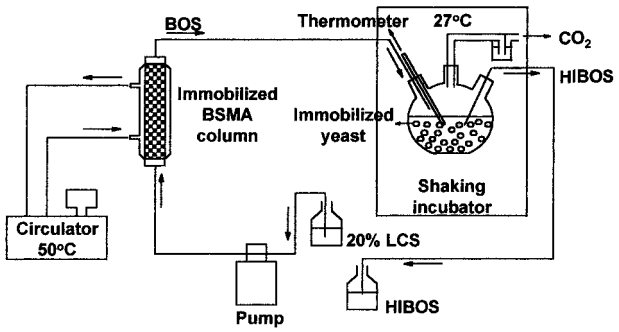

Figure 6. Flow diagram for the production of high-purity BOS: LCS, liquefied corn starch; and HIBOS, high-purity BOS.

ABBREVIATIONS USED

BOS, branched oligosaccharides; BSMA, *B. stearothermophilus* maltogenic amylase; CD, cyclodextrin; DP, degree of polymerization; HPIC, high-performance ion chromatography.

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