Cloning of Novel Maltooligosaccharide-Producing Amylases as Antistaling Agents for Bread

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For better understanding of the antistaling effect of starch-hydrolyzing enzymes, maltose-, maltotriose-, or maltotetraose-producing enzymes were applied to bread mix and the retrogradation rate of the bread was determined using differential scanning calorimetry. A new amylase isolated from *Bacillus subtilis* SUH 4-2, which selectively produces maltose and maltotriose from starch solution (amylase II), and another amylase from *Streptomyces albus* KSM-35, mainly producing maltotetraose and maltotriose (amylase IV), were cloned, characterized, and evaluated as antistaling agents for bread. Addition of amylase II or amylase IV significantly reduced the bread staling rate during 7 days of storage ($p < 0.05$), and especially amylase IV was as effective as a commercial enzyme, Novamyl. Analyses of the maltooligosaccharide composition of bread suggest that maltotriose and maltotetraose produced by the enzyme reaction are responsible for retarding bread retrogradation.

Keywords: *Amylases; antistaling agent; maltooligosaccharide; retrogradation; bread*

INTRODUCTION

Maltooligosaccharides are composed of 2−10 units of α-D-glucopyranose linked by an α−1,4 bond. They have favorable properties such as low sweetness, high water-holding capacity, prevention of sucrose crystallization, and antistaling effect on bread (Park, 1992).

The manufacture of maltooligosaccharides larger than maltotriose had been very difficult, but the discovery of microbial enzymes that produce specific maltooligosaccharides has made it possible to produce syrups containing various maltooligosaccharides (Okada and Nakakuki, 1992). Many amylases producing specific maltooligosaccharide were found: maltohexaose-producing amylases from *Bacillus circulans* G-6 (Takasaki, 1982) and *Bacillus caldovelox* (Fogarty et al., 1991); maltopentaose-producing amylases from *Pseudomonas* sp. (Shida et al., 1992); maltotetraose-producing amylases from *Bacillus* sp. MG-4 (Takasaki et al., 1991), *Streptomyces* sp. KSM-35 (Cha et al., 1994), and *Pseudomonas* sp. IMD353 (Fogarty et al., 1994); maltotriose-producing amylases from *Bacillus subtilis* (Takasaki, 1985); and maltose-producing amylases from *Bacillus licheniformis* (Kim et al., 1992), *Bacillus megaterium* G-2 (Takasaki, 1989), and *Bacillus stearothermophilus* (Outtrup and Norman, 1984).

Staling of baked goods is generally defined as an increase in crumb firmness and corresponding loss in product freshness. The staling is generally accepted to be caused by a gradual transition of starch from an amorphous structure to a partially crystalline state, and the increase in starch crystallinity by an intermolecular or intramolecular association of starch molecules via hydrogen bonds is known as retrogradation (Hebeda et al., 1990). To reduce the retrogradation, many antistaling agents have been studied, and several amylases are used in the baking industry as antistaling agents (Dziezak, 1991; Hebeda et al., 1990, 1991). Martin and Hoseney (1991) reported that high levels of bacterial α-amylase stopped bread firming by producing large quantities of residual dextrin (maltotriose−maltononaose). Boyle and Hebeda (1990) also mentioned that antistaling enzymes not only limit the crystal size but also prevent the starch fraction from readily crystallizing by hydrolyzing branched chains of amylopectin into smaller entities.

For baked goods, it was suggested that enzymes stable in intermediate temperature are suited for the purpose of retarding staling rather than conventional α-amylase (Hebeda et al., 1991). Conventional fungal enzymes have temperature stability in the range of 40−50 °C, which allows inactivation of enzyme before enough hydrolysis of amylopectin occurs. Bacterial enzymes often survive oven temperature, which causes overhydrolyzation of starch and consequently gives gumminess to the final products (Boyle and Hebeda, 1990).

Recently, Yoon et al. (1995) have isolated a novel α-amylase from *B. subtilis* SUH 4-2, which mainly produces maltose and maltotriose from soluble starch solution and has its optimum temperature and pH at 60−65 °C and 5−6, respectively. Maltose and maltotriose had been reported to have antistaling effects on starch gel (Biliaderis and Prokopowich, 1994). The optimum temperature of this α-amylase is in the range

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of intermediate temperature, and it appeared to have a great potential for use as an antistaling agent for baked goods.

Another new amylase was isolated from *Streptomyces albus* KSM-35 by Cha et al. (1994), which had an optimum temperature at 50−60 °C in buffer solution, was stable up to 70 °C in the presence of 10 mM $CaCl_2$, and mainly produced maltotetraose, maltose, and maltotriose from soluble starch solution. The gene encoding the above amylase has been cloned and expressed in *Eschericia coli* (Kim et al., 1996) and *B. subtilis* (Lee et al., 1996b) to increase enzyme production. Because maltotriose or maltotetraose has never been evaluated for retarding bread retrogradation, this enzyme appeared to be suited for this purpose.

This study evaluated the above two maltooligosaccharide-producing amylases as antistaling agents for bread by using differential scanning calorimetry (DSC) measurement as an index of retrogradation.

MATERIALS AND METHODS

Cloning and Production of Amylases. The amylase mainly producing maltose and maltotriose (designated amylase II) was obtained from the culture of *B. subtilis* SUH 4-2 according to the method of Yoon et al. (1995). In brief, the 24-h culture of *B. subtilis* SUH 4-2 was centrifuged, and the obtained supernatant was saturated with 80% ammonium sulfate. The saturated solution was centrifuged at 9000*g* at 4 °C for 25 min, and the obtained pallet was resuspended in 50 mM Tris-HCl buffer and dialyzed against the same buffer at 4 °C for overnight. It was partially purified through a DEAE-Toyopearl and a Mono-Q HR 5/5 column chromatography by using a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The active fractions were collected and concentrated through a YM-10 membrane (Amicon Co., Beverly, MA). Specific activity of the enzyme was increased by 25.5-fold at the final stage.

The amylase mainly producing maltotetraose and maltotriose (designated amylase IV) was originally isolated from *S. albus* KSM-35 (Cha et al., 1994) and sequenced (Lee et al., 1996a). The gene encoding for the enzyme was expressed in *B. subtilis* with the aid of α-amylase-based secretion vector by using the method of Sadaie and Kada (1983) with minor modification (Lee et al., 1996b). The 24-h culture of *B. subtilis* transformant in LB broth was centrifuged, and the proteins in the supernatant were concentrated by precipitating with 80% ammonium sulfate and dialyzed against 20 mM phosphate buffer (pH 7.0) at 4 °C. They were further purified by using DEAE-Toyopearl, Sephadex G-75, and hydroxyapatite columns of an FPLC system. Specific activity of the enzyme was increased by 14.6-fold at the final stage.

Enzyme Characteristics. The hydrolytic activity of amylase II was assayed as described by Kim et al. (1992) with minor modification. The mixture of 0.25 mL of 1% (w/v) soluble starch in 50 mM KH_2PO_4/NaOH buffer (pH 6.0) and 0.2 mL of the same buffer was prewarmed at 60 °C for 5 min. The 0.05 mL of enzyme solution was added to the prewarmed solution and incubated for 30 min. The reaction was terminated by adding 1.5 mL of dinitrosalicylic acid solution (Miller, 1959). The solution was boiled for 5 min in a water bath and cooled under running tap water, and the absorbance was measured at 575 nm. One unit of activity was defined as the amount of enzyme producing 1 mol of reducing sugar (with glucose as standard)/min. The activity of amylase IV was assayed according to the same method used above except the reaction temperature was 50 °C.

To analyze the change of sugar composition of hydrolyzed starch solution, the above reaction mixture was incubated for up to 24 or 48 h at each optimum temperature, treated with acetonitrile, centrifuged, filtered through a 0.45 μm filter, and analyzed by high-performance liquid chromatography (HPLC), performed under the following conditions: column, LiChrosorb NH_2 column (5 μm, 4.6 × 250 mm, Merck); eluent, acetonitrile/distilled water (70:30, v/v); detector, RI detector (Waters R400); flow rate, 1 mL/min; sample loaded, 20 μL.

Antistaling Effect of Amylases on Bread. Four groups of bread were baked in an automatic home breadmaker (SHB-600, Samsung Co., Korea) using White Pan Bread Mix II (Cheiljedang Co., Korea) and stored at 4 °C in polyethylene bags. The formulation of bread mix II was as following: 100 parts of white flour, 8.9 parts of sugar, 5.2 parts of shortening, 2.2 parts of defatted milk powder, 0.1 part of yeast, 1.4 parts of salt, and 69 parts of water (parts by weight). The four groups were control, supplemented with 412 IU of amylase II, supplemented with 1030 IU of amylase IV, and supplemented with 0.02% (w/w) Novamyl (Novo Nordisk, Denmark), a commercial antistaling maltogenic amylase. The amount of enzyme supplementation was predetermined by sensory evaluation in which the texture, volume, and appearance of breads were considered for optimum quality at various enzyme levels.

Five loaves of bread were baked for each group, except four for the group supplemented with 412 units of amylase II. To estimate the retrogradation rate, DSC was performed as described by Kweon et al. (1994) at 1, 3, and 7 days after baking by using DSC 120 (Seico Co., Japan) calibrated with indium and zinc. Distilled water was used as a reference material. Bread samples (∼10 mg) were weighed and hermetically sealed in aluminum pans. The pans were heated from 20 to 130 °C at 5 °C increase/min. The enthalpy of each bread (joules per gram) was determined by the first endothermic peak area, and the enthalpy, the energy required to melt the crystalline material, was used as the index of retrogradation (Kweon et al., 1994).

Maltooligosaccharide Composition of Breads. To measure the differences in maltooligosaccharide composition among control and amylase-treated breads, HPLC was performed as described by Akers and Hoseney (1994) with minor modification. Ten grams of bread crumb and 100 mL of distilled water were mixed and stirred vigorously for 1 h at room temperature. The mixture was centrifuged at 10000*g* for 10 min, and the supernatant was diluted with acetonitrile (1:1, v/v). Insoluble fractions of diluted solution were discarded again by centrifugation at 12500*g* for 5 min, and the supernatant was filtered through a 0.45-μm filter (Gelman Sciences). HPLC analysis was performed under the same conditions described above.

Statistical Analysis. Statistical analyses of data were performed using PC-SAS (SAS Institute).

RESULTS AND DISCUSSION

Hydrolysis Pattern of Starch by Amylase II and Amylase IV. As the incubation time increased, different patterns of oligosaccharide composition were observed for each enzyme. For amylase II, the contents of maltose and maltotriose dominated others from an early stage of the reaction. The content of maltotriose was high at the beginning; however, it gradually decreased, and that of glucose was maintained at ∼1 mg/mL throughout the incubation period. The 24-h hydrolysis product was composed of glucose, maltose, maltotriose, and maltotetraose in a ratio of 11:59:25:5 (Figure 1a).

The amount of amylase IV produced from *B. subtilis* transformant was ∼27-fold greater than that of the parental strain, *S. albus* KSM-35. They had the same characteristics as those from the parental strain, i.e. optimum pH and temperature were 6 and 50−60 °C, respectively. Although <60% of hydrolysis product was maltotetraose in the early stage of reaction, as the reaction proceeded, the content of maltotetraose was gradually decreased (Figure 1b).

Antistaling effect of Amylase II and Amylase IV on Bread. There were significant differences ($p < 0.05$) in bread retrogradation rates depending upon the type

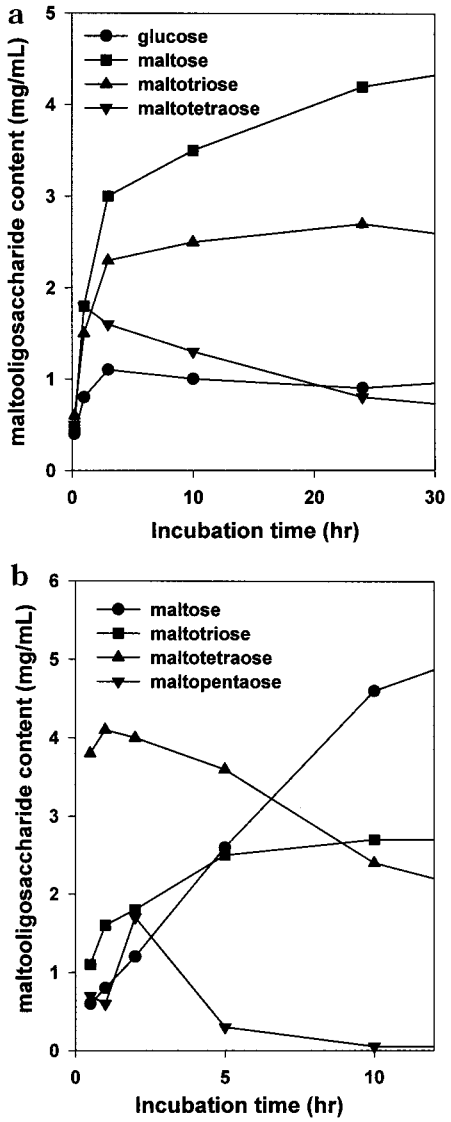

Figure 1. Change of maltooligosaccharide composition of soluble starch solution treated with amylase II (a) or amylase IV (b) as the incubation time increased.

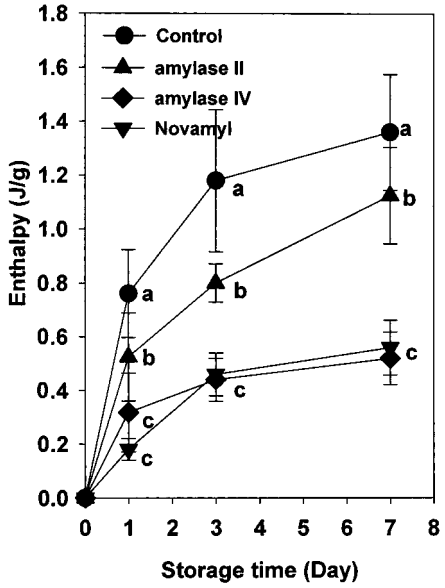

Figure 2. Retrogradation rates of the breads treated with various enzymes during storage. Values with different letters in each storage time are significantly different ($p < 0.05$).

Table 1. Comparison of Maltooligosaccharide Composition of Breads Treated with Different Enzymes by Using HPLC

enzyme treated	composition[a] (mg/g of crumb) maltose	maltotriose	malto-tetraose	malto-pentaose
control	23.6 ± 0.71^{b}	0	0	0
amylase II	29.1 ± 0.64^{a}	1.95 ± 0.23^{c}	0	0
amylase IV	24.2 ± 0.75^{b}	4.60 ± 0.34^{a}	1.31 ± 0.53^{b}	1.35 ± 0.31
Novamyl	22.2 ± 0.07^{c}	3.76 ± 0.08^{b}	4.23 ± 0.12^{a}	0

[a] Values with different letters in each column are significantly different ($p < 0.05$).

of amylase supplemented (Figure 2). Addition of amylase IV to bread mix gave significantly lower retrogradation rates to the breads throughout the storage period ($p < 0.05$), which was as effective as the addition of Novamyl. Compared with control, the breads supplemented with amylase II also showed significantly retarded retrogradation ($p < 0.05$); however, the effect was lower than with amylase IV or Novamyl.

Maltooligosaccharide in Bread. All groups of bread treated with amylase had increased content of maltooligosaccharides ranging from 24 to 31 mg/g of bread crumb (Table 1). Major maltooligosacchrides produced were maltose, maltotriose, and maltotetraose. Depending upon the type of enzyme added, the compositions of maltooligosaccharide in bread were different.

A significant amount of maltose was produced in breads without any enzyme treatment, which is believed to be from yeast fermentation or hydrolysis of starch molecules during the heat process of baking. The fact that no other maltooligosaccharide except maltose was produced in control bread suggests that maltotriose, maltotetraose, or maltopentaose found in bread was produced by the action of amylase II or amylase IV. The breads supplemented with amylase II had some maltotriose and a significantly high amount of maltose, which was in accord with the results of soluble starch hydrolysis. The supplement of amylase IV produced maltose, maltotriose, maltotetraose, and maltopentaose in bread, which is generally in accord with the pattern of starch hydrolysis. The slight difference in maltooligosaccharide composition could be due to the low moisture content of substrate and the high-temperature condition in baking. Treatment of Novamyl mainly produced maltotriose and maltotetraose. Maltotetraose once was reported to have relatively little influence on reducing retrogradation of starch gel (Biliaderis and Prokopowich, 1994); however, it appeared to be effective in retarding bread retrogradation (Table 1). From the results that the breads supplemented with amylase IV or Novamyl showed significantly slower rates of retrogradation compared with control or amylase II, their retention of maltotriose and maltotetraose suggests that these sugars are responsible for retarding bread retrogradation. On the contrary, the formation of maltose as in amylase II supplementation seemed to have little effect.

The antistaling effect by amylase IV or Novamyl in this study could be partially explained by the results of Donnelly et al. (1973), who found that maltotriose and maltotetraose showed superior hygroscopicities compared with other maltooligosaccharides. Consequently, they would have exerted antistaling effect by better holding water molecules surrounding starch molecules and inhibiting the starch−starch interaction. On the

contrary, maltose was reported as the least hygroscopic maltooligosaccharide (Donnelly et al., 1973). Martin and Hoseney (1991) postulated the antistaling mechanism of bread as dextrins of a particular size produced by enzyme reaction during baking interfered with the interactions, possibly by hydrogen bond, between starch molecules and the continuous protein matrix, gluten. Under this mechanism, maltotriose and maltotetraose are believed to be the right size of dextrin to interfere with interactions, and maltose might be less effective for preventing bread retrogradation because maltose is a relatively small molecule and may diffuse easily.

The importance of dextrins produced by enzyme reaction for bread retrogradation was reconfirmed by Akers and Hoseney (1994). They suggested indirectly that dextrins of size greater than maltoheptaose have an antifirming effect on bread. The molecular size of starch in the bread mix must have been reduced by the hydrolysis of amylase II or amylase IV, and the resulting modified starch could also exhibit an antistaling effect on bread. Therefore, our results are not considered in contradiction to them. Moreover, they did not evaluate the effect of small size of maltooligosaccharide and, as they mentioned, the extent of preventing bread retrogradation would be significantly different depending upon the type of enzyme used. Future study on the direct supplementation of various sizes of maltooligosaccharides in bread mix would explain the mechanism, although the heat effect during baking should also be considered at the same time.

In conclusion, both amylase II and amylase IV showed great potential as antistaling agents for bread, and maltotriose and maltotetraose produced by these enzymes appeared to be responsible for preventing bread retrogradation.

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