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Purification and Characterization of Branching Specificity of a Novel Extracellular Amylolytic Enzyme from Marine Hyperthermophilic *Rhodothermus marinus*

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An extracellular enzyme (RMEBE) possessing α-(1→4)-(1→6)-transferring activity was purified to homogeneity from *Rhodothermus marinus* by combination of ammonium sulfate precipitation, Q-Sepharose ion-exchange, and Superdex-200 gel filtration chromatographies, and preparative native polyacrylamide gel electrophoresis. The purified enzyme had an optimum pH of 6.0 and was highly thermostable with a maximal activity at 80°C. Its half-life was determined to be 73.7 and 16.7 min at 80 and 85°C, respectively. The enzyme was also halophilic and highly halotolerant up to about 2 M NaCl, with a maximal activity at 0.5 M. The substrate specificity of RMEBE suggested that it possesses partial characteristics of both glucan branching enzyme and neopullulanase. RMEBE clearly produced branched glucans from amylose, with partial α-(1→4)-hydrolysis of amylose and starch. At the same time, it hydrolyzed pullulan partly to panose, and exhibited α-(1→4)-(1→6)-transferase activity for small maltooligosaccharides, producing disproportionated α-(1→6)-branched maltooligosaccharides. The enzyme preferred maltopentaose and maltohexaose to smaller maltooligosaccharides for production of longer branched products. Thus, the results suggest that RMEBE might be applied for production of branched oligosaccharides from small maltodextrins at high temperature or even at high salinity.

Keywords: *Rhodothermus marinus*, extracellular enzyme, hyperthermophilic enzyme, α-(1→6)-branching activity, branched maltooligosaccharides

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Rhodothermus marinus is a Gram-negative marine eubacterium originally isolated from an alkaline submarine hot spring [1, 16, 21]. It is obligately aerobic and moderately halophilic with optimum growth around 65°C and 2% NaCl. It has been known to produce various kinds of thermostable enzymes, particularly polysaccharide-degrading enzymes that are expected to have advantages in industrial processes, including β-glucanase, cellulase, xylanase, α-L-arabinofuranosidase, and chitinase [3, 9, 10, 15, 22]. Most of those enzymes were found extracellularly and exhibited optimal activity at about 80–85°C.

Various hyperthermophilic amylolytic enzymes are of great interest because of their efficient starch liquefaction and saccharification at high temperatures, as well as their extreme stability, low viscosity, and reduced microbial contamination in a reaction medium at high temperatures [2, 6, 24]. Reportedly, extracellular amylase and pullulanase that are highly thermostable have been found in culture broth of *R. marinus*, which had half-lives of 3 h and 30 min at 85°C, respectively [7]. An intracellular thermostable branching enzyme from *R. marinus* was also cloned and characterized to have a half-life of 16 h at 80°C [20]. However, there exist no more reports on other starch-degrading or -modifying enzymes from this microorganism. We have recently detected amylolytic and concurrent glucan-transferring activities in the culture supernatant of *R. marinus*. Therefore, in this study, we purified the extracellular enzyme that exhibited hydrolyses of starch and maltooligosaccharides and simultaneous transfer of hydrolyzed glucans, and characterized the catalytic properties in terms of branching specificity, with optimal reaction conditions.

Materials and Methods

Microorganism and Culture Conditions

R. marinus was obtained from the American Type Culture Collection (VA, U.S.A.) and cultivated in Marine Broth 2216 (Difco, NJ, U.S.A.) at 70°C and initial pH 7.0 for 3 days with shaking (160 rpm), as described previously [7]. The amylose, soluble starch, maltooligosaccharides, pullulan, isoamylase, and β-amylase were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All the other chemicals used were of reagent grade.

Purification of Extracellular Enzyme

Cells were harvested by centrifugation (7,000 ×*g* for 15 min at 4°C). The resulting culture supernatant was collected as a crude enzyme solution, and ammonium sulfate (80% saturation) was added to precipitate the enzyme. The precipitate of crude enzyme was dissolved in a 50 mM Tris-HCl buffer (pH 8.0), dialyzed against the same buffer for 12 h at 4°C, and then concentrated by ultrafiltration (Millipore Co., Bedford, MA, U.S.A.) for further purification. The concentrated protein sample was loaded onto a Q-sepharose column preequilibrated with 50 mM Tris-HCl buffer (pH 8.0), and eluted with a NaCl gradient of 0–1 M in the same buffer at a flow rate of 2 ml/min. Active fractions that exhibit amylolytic and transglycosylating activities were pooled and concentrated by ultrafiltration. Successively, gel filtration with a Superdex 200 column was performed with an elution by 50 mM sodium acetate buffer (pH 6.0) containing 150 mM NaCl at 0.4 ml/min. Active fractions exhibiting transglycosylating activity were collected, concentrated, and further purified by native polyacrylamide gel electrophoresis (PAGE).

Active sample was subjected to native PAGE with 12.5% (w/v) acrylamide separating gel and 5% (w/v) stacking gel. The electrophoresis was carried out at a constant voltage of 60 V for 4 h using a Tris-glycine buffer (pH 8.8) except 0.1% SDS. Zymogram analysis was performed using a part of the gel and confirmed the existence of a clear zone containing an active enzyme [27]. After cutting the gel, the active enzyme was eluted in 10 mM sodium acetate buffer (pH 6.0) by incubation in a shaking water bath (40°C, 120 rpm), and concentrated for further investigation. The protein concentration was determined according to the Bradford method [4], with bovine serum albumin as a standard. The purity and molecular mass of the purified protein were estimated by sodium dodecyl sulfate (SDS)-PAGE using a 10% (w/v) acrylamide gel [19]. The gel was stained for protein with Coomassie Brilliant Blue R solution [45% (v/v) methanol, 10% (v/v) acetic acid] and destained with acetic acid:methanol: distilled water (1/1/8, v/v/v).

Zymogram Analysis

After SDS-PAGE, zymogram analysis of purified enzyme was performed on the gel [27]. The gel was soaked in 0.5% (w/v) Triton X-100 solution to remove SDS for 30 min with gentle agitation. After washing the gel twice, it was incubated in 1% (w/v) soluble starch in 50 mM sodium acetate buffer (pH 6.0) at 70°C for 1 h. The gel was then washed with distilled water and finally stained with iodine solution [50% (v/v) methanol, 1% (w/v) iodine, and 10% (w/v) potassium iodide] for 5 min at room temperature. The appearance of a clear zone on a dark-blue background was associated with the enzyme activity.

Enzyme Assay

The enzyme activity was assayed at 70°C by using the dinitrosalicylate (DNS) method with a slight modification for the determination of reducing sugars [13]. Maltose was used as a standard sugar for the DNS assay. A reaction mixture in a standard condition was composed of 50 µl of 1% (w/v) soluble starch in 50 mM sodium acetate buffer (pH 6.0), 40 µl of the same buffer, and 10 µl (2–20 units) of enzyme solution. The reaction was stopped and colored by adding 300 µl of the DNS solution (10.6 g of 3,5-dinitrosalicylic acid, 19.8 g of NaOH, 306 g of sodium potassium tartrate, 7.7 ml of phenol, 8.3 g of sodium metabisulfite, and 1,416 ml of water), followed by boiling for 5 min. After cooling, the absorbance of the reaction mixture was measured at 575 nm by a VERSA max microplate reader (Molecular Device Corporation, CA, U.S.A.). One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of maltose per min under the assay condition.

Effects of pH and Temperature on Enzyme Activity and Stability

To determine the optimal pH for enzyme activity and pH range for enzyme stability, the hydrolytic activity of the purified enzyme was examined using soluble starch in the following 50 mM buffers with various pHs: sodium acetate (pH 3.0 to 6.0), sodium phosphate (pH 6.0 to 7.0), and Tris-HCl (7.0 to 9.0). To examine the pH stability, the enzyme was first incubated in the buffers with various pHs for 1 h at 25°C, and then the remaining activity was measured under the standard conditions described above. The optimal temperature for the purified enzyme activity was determined in 50 mM sodium acetate buffer (pH 6.0) using a range from 40 to 90°C. The thermal stability of the purified enzyme in the same buffer (pH 6.0) was analyzed by incubating the enzyme solution (0.1 mg/ml) at different temperatures (75, 80, and 85°C), from which aliquots were taken at various time points and placed immediately in an ice-water bath. The residual activities of the aliquots were determined under the standard conditions.

Effects of Salt Concentration, Metal Ions, and Organic Solvents on Enzyme Activity

The effect of salt concentration on the enzyme activity was measured in the standard buffer (pH 6.0) containing 0–4 M of NaCl. The effects of metal ions and organic solvents were also investigated using $MnCl_2$, $CuSO_4$, $FeCl_2$, $ZnCl_2$, $MgCl_2$, $CaCl_2$, $BaCl_2$, and LiCl, plus dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol, and ethanol, respectively. The enzyme was incubated in the absence and presence of 5 mM cations or a 10% (v/v) organic solvent in the standard buffer for 10 min at 25°C. Immediately after the pre-incubation, an appropriate aliquot was taken and the relative activity was measured under the standard conditions.

Enzyme Incubation with Starch and Maltooligosaccharides

A substrate solution (0.5%, w/v) of soluble starch or amylose was prepared by dissolving in 1 N NaOH (6 ml), followed by the addition of demineralized water (15 ml) and 200 mM sodium acetate buffer (3 ml, pH 6.0) [25]. The pH was adjusted to 6.0 with 1 N HCl (6 ml). Each substrate solution (0.5%, w/v) was gelatinized by incubation at 100°C for 30 min and then incubated with RMEBE for 12 or 24 h at 70°C. Maltooligosaccharides of maltose (G2) to maltohexaose (G6) were reacted in the standard buffer (pH 6.0) with the purified enzyme for 3–12 h at 70°C. The reaction was stopped by addition of 1 N HCl and analyzed by thin-layer chromatography

(TLC) on Whatman K5F silica gel plates (Whatman, Kent, U.K.) with isopropyl alcohol:ethyl acetate:water (3/1/1, v/v/v) [17]. After irrigating once or twice, the TLC plate was dried and visualized by dipping in a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and heating at 110°C for 10 min.

Analysis of Chain Length Distribution

After the enzyme incubation (12 h at 70°C) with the amylose substrate (0.5%, w/v) as described above, three volumes of ethanol was added to the quenched reaction mixture and the mixture then stored for 1 h at 4°C. The resulting precipitate was collected by centrifugation (10,000 ×*g*, 10 min) and washed three times with 70% ethanol, followed by vaccum drying. The dried product (10 mg) was dissolved in 1 ml of a 250 mM sodium acetate buffer (pH 3.5) and then incubated with 20 μl of isoamylase (0.5 U/μl, Sigma Chemical Co.) for 24 h at 37°C, followed by boiling for 5 min. After centrifugation of the reaction mixture, a supernatant containing debranched glucan chains was taken and subjected to filtration with a 0.22 μm membrane filter (Millipore, Billerica, MA, U.S.A.). The distribution of branch chain-length was then analyzed by high-performance anionic-exchange chromatography (HPAEC), using a Dionex CarboPac PA100 column (0.4×25 cm, Dionex Co., Sunnyvale, CA, U.S.A.) and an electrochemical detector ED40 (Dionex Co.) with a linear gradient of sodium acetate from 0 to 850 mM for 60 min and 150 mM sodium hydroxide at a flow rate of 1.0 ml/min [11].

Analysis of Branched Products

The product mixture of enzyme reaction with 1% (w/v) G5 in the standard buffer (pH 6.0) was treated by β-amylase (10 U) for 24 h at 37°C, and then analyzed by TLC. The glucan products from the RMEBE reaction with G5 were isolated by preparative paper chromatography on a Whatman 3 MM paper (23×55 cm) with a descending technique [18]. The spots on the paper were located using a $AgNO_3$ reagent to verify the separation of purified carbohydrates. The paper was sectioned and eluted with deionized water, and then lyophilized for the analysis. The molecular weights of the purified products were determined by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a Voyager-DE MALDI-TOF MS (Perseptive Biosystems, Framingham, MA, U.S.A.) in linear mode, in which α-cyano-4-hydroxy cinnamic acid (Sigma Chemical Co.) was used as a matrix [26].

RESULTS AND DISCUSSION

Purification of Extracellular Transferase

The culture broth of *R. marinus* in this study exhibited an amylolytic activity. By this enzyme activity, soluble starch was hydrolyzed to produce smaller maltooligosaccharides and increase reducing sugars. This result agrees with the previous report that *R. marinus* is capable to extracellularly produce amylase and pullulanase, which hydrolyze starch [7]. When maltopentaose (G5) was tested as a substrate in this study, it was first noted that a transglycosylation activity was detected in the culture broth to produce a higher degree of polymerization (DP) of maltooligosaccharides than G5. The enzyme possessing the transglycosylating activity was sought in the present study and successfully purified by combination of 80% ammonium sulfate precipitation, Q-Sepharose ion-exchange, and Superdex-200 gel filtration chromatographies, and preparative native PAGE. When the culture sample was eluted from the Q-Sepharose column, an active protein peak was found to have both the amylolytic activity for starch and the transferring activity for G5, even though other protein peaks were eluted to have only the amylolytic activity (data not shown). The active fractions with the transferring activity were pooled and applied onto a Superdex-200 column consequently to get a highly purified active protein fraction. Moreover, the resulting active fraction was applied to native PAGE, and the active protein in the zymogram was finally purified to homogeneity by elution from the native PAGE gel (Fig. 1). The purified enzyme that possessed both amylolytic and transferring activities showed a single protein band on SDS-PAGE gel with an estimated molecular mass of approximately 66 kDa.

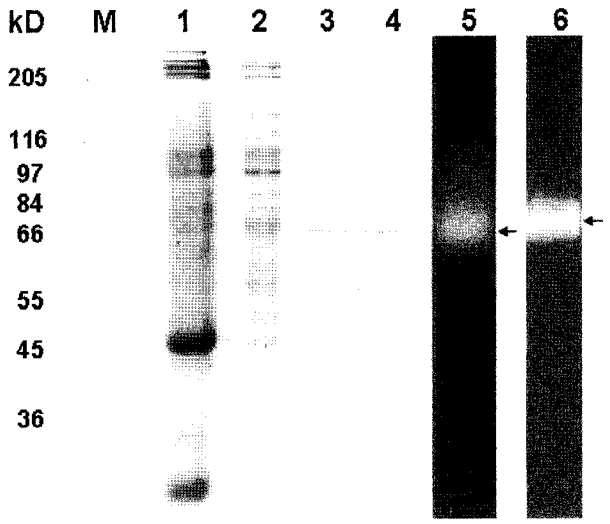

Fig. 1. SDS-PAGE analysis of purified RMEBE at different stages of purification.

Lane M, protein size standards; lane 1, ammonium sulfate precipitation of culture supernatant; lane 2, Q-Sepharose column; lane 3, purified from Superdex 200 column chromatography; lane 4, purifed RMEBE from extraction of native PAGE band; lane 5, zymogram of purified RMEBE (arrow) in SDS-PAGE; lane 6, zymogram of purified RMEBE (arrow) in native PAGE.

Amylolytic and Branching Catalytic Properties of Purified Enzyme

The action pattern of purified enzyme was investigated with various substrates typically used for analyzing amylolytic enzymes, including soluble starch, amylose, maltooligosaccharides, and pullulan. The purified enzyme hydrolyzed soluble starch to produce various DPs of linear and branched maltooligosaccharides larger than G2 and glucose, and also hydrolyzed pullulan partly to panose and isomaltose (lanes 2 and 6 in Fig. 2). This indicated that the

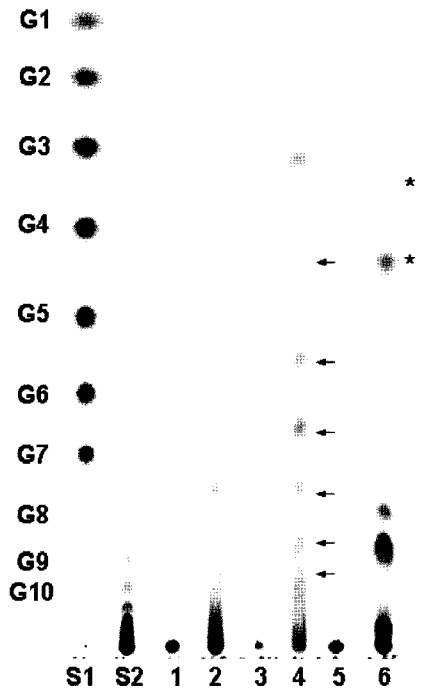

Fig. 2. TLC analysis of RMEBE reaction with various glucan substrates.
Lane S1, maltooligosaccharide standards (G1–G7); lane S2, maltooligosaccharide standards (G1–G20) prepared from soluble starch by β-amylase; lane 1, soluble starch; lane 2, reaction of RMEBE with soluble starch; lane 3, amylose; lane 4, reaction of RMEBE with amylose; lane 5, pullulan; lane 6, reaction of RMEBE with pullulan. The reaction of RMEBE was carried out at 70°C for 12 h. Arrows in lane 4 indicate maltooligosaccharides of branched structure and the asterisks in lane 6 indicate isomaltose and panose, respectively.

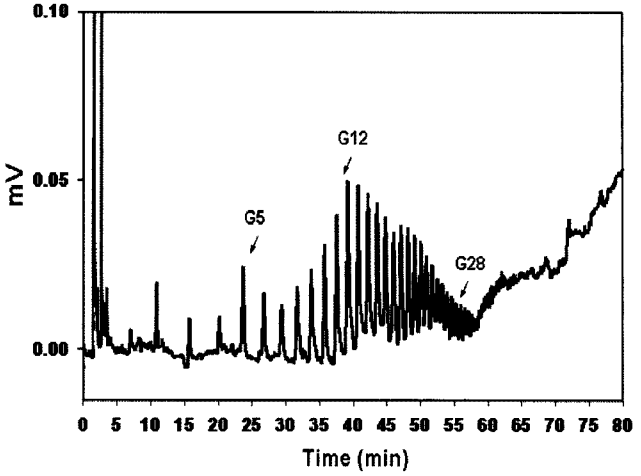

Fig. 3. HPAEC analysis on the side chain distribution of the branched product from RMEBE reaction with amylose.
The amylose was incubated with RMEBE at 70°C for 12 h. The glucan product was isolated by ethanol precipitation, treated with isoamylase, and eluted through a CarboPac PA100 column. G5, G12, and G28 indicate peaks of maltooligosaccharides with degree of polymerization of 5, 12, and 28 glucoses, respectively.

enzyme had an endo-acting hydrolyzing activity for α-(1→4)-glucosidic linkages of starch and pullulan, which is partly similar to *Thermoactinomyces vulgaris* α-amylase (TVA I) activity. TVA I, an extracellular enzyme, hydrolyzes starch and pullulan to small maltooligosaccharides and panose, respectively [23]. Interestingly, this purified enzyme hydrolyzed amylose to small linear maltooligosaccharides and simultaneously produced branched maltooligosaccharides (arrows in lane 4 of Fig. 2). The amylose used was virtually free of α-(1→6)-branch linkage. The formation of branched maltooligosaccharides suggested that the purified enzyme had an α-(1→4)-(1→6)-transferase activity as well as the α-(1→4)-hydrolysis activity for amylose. In fact, this activity is not detected in TVA I. It seemed to be closely common in the activity of bacterial branching enzyme (BE), which exhibits a branching activity forming α-(1→6)-glucosidic linkage immediately after α-(1→4)-cleavage [8]. BE degrades starch or amylose to small cyclic dextrins with α-(1→4)-hydrolyzed short-chain glucans [14, 25]. Then, we purified the reaction product formed from amylose by the purified enzyme and analyzed the branch chain composition of the product by HPAEC after isoamylase debranching, as described above. The result confirmed that the purified enzyme had the α-(1→6)-branching activity for amylose, producing branched glucan with a series of side chains (Fig. 3). It showed a general branch chain-length distribution, in which the optimum was DP 12 and the profile range was up to approximately DP 28. Thus, this purified enzyme was supposed to be a novel extracellular branching enzyme from *R. marinus* (RMEBE).

In addition, RMEBE showed unusual substrate specificity. The enzyme employed small maltooligosaccharides (G3–G6) to hydrolyze and transfer a maltooligosyl chain to other maltooligosaccharide molecules (Fig. 4). Because of this transglycosylation activity, RMEBE produced disproportionated products, smaller and larger DPs of maltooligosaccharides than the substrate used. This disproportionation was clearly observed for G5 or G6 substrate (lanes 10 and 12 in Fig. 4). Among the hydrolyzed products from the substrate, G2 and G3 occupied a much higher amount. Until now, there has been no report on this kind of transglycosylation activity for small maltooligosaccharides by a branching enzyme. From the RMEBE reaction with G5, we purified major transglycosylation products larger than the substrate (inset lane 5 in Fig. 5) and determined the molecular masses (Fig. 5). From the mass analysis of the major purified maltooligosaccharides (arrows in inset lane 4), two major peaks clearly appeared at *m/z* 1175.4 and 1337.5 ($[M+Na]^+$), which respectively corresponded to the calculated molecular masses of sodium ion adducts of maltoheptaose (G7) and maltooctaose (G8). Two purified products exhibited slower mobility on upward TLC than the same molecular weight (M_w) of linear α-(1→4)-linked maltooligosaccharides, G7 and G8, respectively. This is generally due to the existence of α-(1→6)-linkage in the compounds. Panose

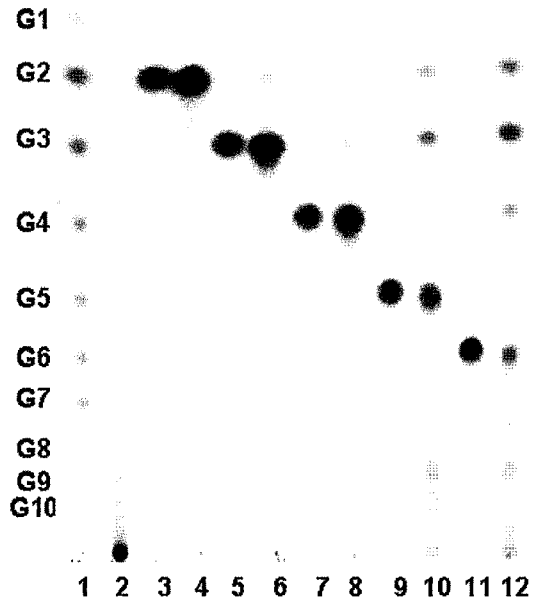

Fig. 4. TLC analysis of RMEBE reaction with various small maltooligosaccharides.

Lane 1, maltooligosaccharide standards (G1–G7); lane 2, maltooligosaccharide standards (G1–G20); lane 3, maltose; lane 4, reaction of RMEBE with maltose; lane 5, maltotriose; lane 6, reaction of RMEBE with maltotriose; lane 7, maltotetraose; lane 8, reaction of RMEBE with maltotetraose; lane 9, maltopentaose; lane 10, reaction of RMEBE with maltopentaose; lane 11, maltohexaose; lane 12, reaction of RMEBE with maltohexaose. The reaction of RMEBE was carried out at 70°C for 3 h.

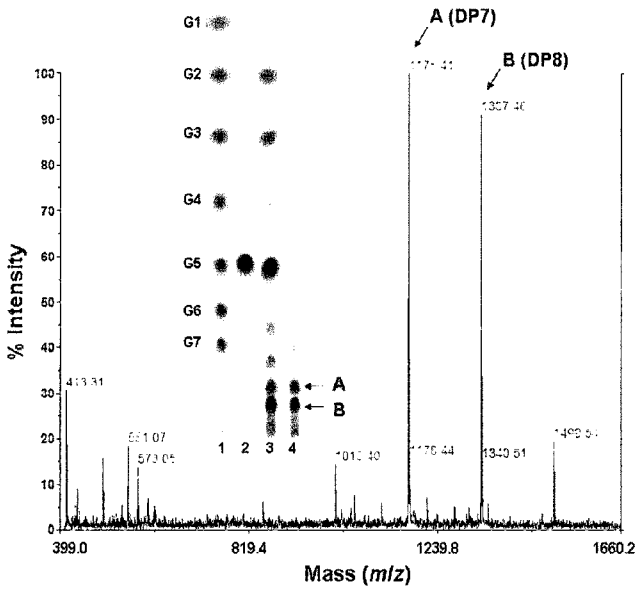

Fig. 5. Molecular mass determination of purified products from RMEBE reaction with maltopentaose.

The purified products are shown in lane 4 of the inset TLC panel. The molecular masses were determined by MALDI-TOF mass analysis. Lane 1, maltooligosaccharide standards (G1–G7); lane 2, maltopentaose; lane 3, reaction of RMEBE with maltopentaose; lane 4, purified products by paper chromatography. A (DP7) and B (DP8) indicate peaks of maltooligosaccharides with degree of polymerization of 7 and 8 glucoses, respectively, which correspond to A and B designated in the inset panel.

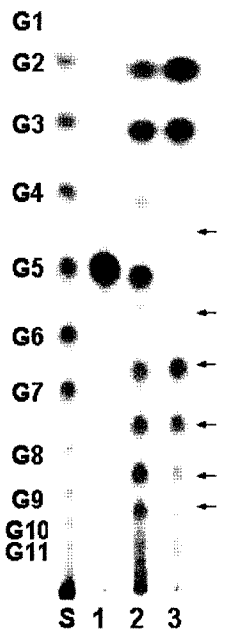

Fig. 6. TLC analysis of RMEBE reaction products from maltopentaose after β-amylase treatment.

Lane S, maltooligosaccharide standards (G1–G20); lane 1, maltopentaose; lane 2, RMEBE reaction with maltopentaose; lane 3, β-amylase reaction with products in lane 2. Arrows indicate transfer products produced by the α-(1→6)-transferring activity of RMEBE.

containing α-(1→6)-linkage exhibits slower mobility than the same M_w of linear α-(1→4)-linked maltotriose in the same manner [5]. The α-(1→6)-branched maltooligosaccharides (arrows in lane 3 of Fig. 6) remained after β-amylase treatment. The resulting branched maltooligosaccharides then disappeared by α-(1→6)-cleaving isoamylase treatment and only maltooligosaccharides from G2 to G5 mainly remained (data not shown).

Accordingly, this result confirmed that RMEBE also had α-(1→4)-(1→6)-transferase activity for small maltooligosaccharides, producing α-(1→6)-branched maltooligosaccharides. In fact, this activity pattern was partly similar to neopullulanase. It hydrolyzes pullulan to panose, and employs small maltooligosaccharides to produce isomaltooligosaccharides by α-(1→4)-(1→6)-transferase activity [12]. However, neopullulanase also catalyzed α-(1→6) hydrolysis as well as α-(1→4)-hydrolysis with α-(1→4)-(1→4)-transglycosylation. As a result, RMEBE possessed both partial activities of glucan branching enzyme and neopullulanase. Conclusively, RMEBE exhibited α-(1→4)-(1→6)-branching activity for both low M_w maltooligosaccharides and high M_w amylose, which has unique branching specificity as a branching enzyme.

Dependence of RMEBE Activity on Environmental Reaction Conditions

The pH range at which RMEBE remained active and stable was determined using soluble starch as the substrate.

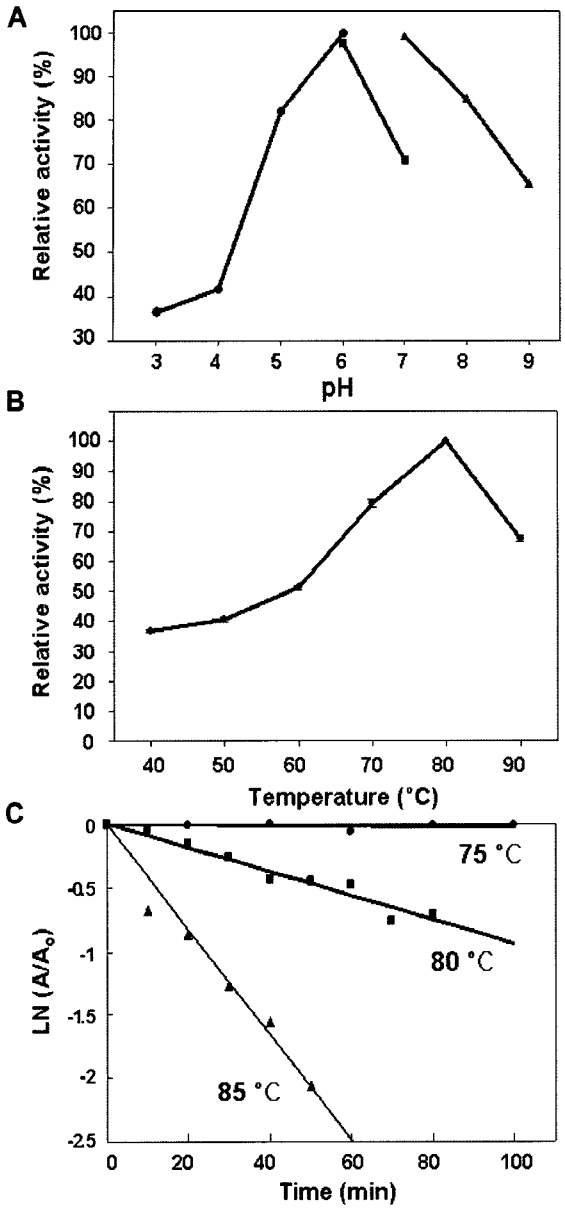

Fig. 7. Optimum pH (**A**) and temperature (**B**) for activity and thermal inactivation (**C**) of RMEBE.
A. Values in ordinate are shown as percentages of maximum specific activity (100%) observed at pH 6.0. **B**. Values are shown as percentages of specific activity (100%) observed at 80°C. **C**. LN (A/A_0) means logarithm of the ratio of relative activities between a given time and a zero time at each temperature.

Table 1. Relative activity of RMEBE in the presence of high NaCl concentration.

NaCl (M)	Relative activity (%)
0	100
0.5	112±1.0
1.0	108±4.3
2.0	99±1.8
3.0	86±4.6
4.0	71±8.4

Reaction was carried out in the absence or presence of sodium chloride at final concentration of 0.5–4 M.

The maximum RMEBE activity was observed at pH 6.0 (Fig. 7A), while more than 70% of the maximum activity was maintained for 12 h incubation in the range between pH 5 and 8. The optimal temperature for the enzyme was about 80°C (Fig. 7B). Generally, *R. marinus* grew at 55–85°C [3]. As expected, the enzyme exhibited a remarkable thermal stability, retaining its full activity after 100 min of incubation at 75°C (Fig. 7C). The half-life of the enzyme was determined to be 73.7 and 16.7 min at 80 and 85°C, respectively. When compared with previously reported *R. marinus* amylase in crude culture solution [7], RMEBE showed lower optimum pH by 0.5–1 units, and similar optimal temperature and thermostability.

The activity of RMEBE was measured in the presence of NaCl ranging from 0 to 4 M (Table 1). The activity was a maximum at 0.5 M NaCl and the initial activity in the absence of NaCl was almost fully maintained up to 2 M. RMEBE still retained approximately 70% of the initial activity at 4 M NaCl. Reportedly, *R. marinus* grew at NaCl concentration ranging from 0.085 to over 1 M with an optimal growth at 0.34 M [3]. Therefore, it was reasonable that this extracellular enzyme showed halophilic or halotolerant property, because it would be excreted to a high-salinity environment. In addition, the RMEBE activity was slightly activated or inhibited by 5 mM metal ions tested (Table 2). Among them, Mg^{2+} increased the enzyme activity by 15% compared with the original activity, whereas Mn^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} decreased the activity by 16–62%. The enzyme activity was also inhibited by 44% and 23–65% in the presence of 5 mM EDTA and 10% (v/v) organic solvents, respectively.

In conclusion, the extracellular branching enzyme of approximately 66 kDa from *R. marinus* was highly

Table 2. Effect of metal ions and organic acids on RMEBE activity.

Metal ions or organic solvents	Relative activity (%)
None	100
Mn^{2+}	46.5±0.4
Cu^{2+}	83.6±0.6
Fe^{2+}	41.7±0.7
Zn^{2+}	38.2±1.3
Mg^{2+}	115.4±2.5
Ca^{2+}	96.4±1.6
Ba^{2+}	97.5±0.4
Li^{+}	100.3±2.0
EDTA[a]	55.8±1.7
DMSO	77.1±0.3
DMF	35.0±1.0
Methanol	54.8±1.1
Ethanol	42.8±0.5

Reaction was carried out in the absence or presence of metal ions at final concentration of 5 mM or 10% organic solvents (v/v), respectively.
[a]EDTA (5 mM) was added as a chelating agent.

thermostable and halotolerant. This RMEBE exhibited α-(1→4)-(1→6)-branching activity when either low M_w maltooligosaccharides or high M_w amylose was employed as a substrate. RMEBE might be applied to produce branched maltooligosaccharides from small maltodextrins or starch by its unique branching activity at high temperature and, if needed, at high salinity.

Acknowledgments

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