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Characterization and mechanism of action of *Microbacterium imperiale* glucan 1,4-α-maltotriohydrolase

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ABSTRACT

In this study, glucan 1,4-α-maltotriohydrolase (AMTS) from *Microbacterium imperiale* was purified and characterized. Hydrolysis by AMTS was affected by starch structure (e.g., amylose versus amylopectin) and hydrolysis time. During the initial phase of hydrolysis of maltooligosaccharides (G4–G7), AMTS displayed a unique transfer specificity to the transfer of maltotriosyl units. After extensive hydrolysis, maltotriose became the major end product, followed by glucose and maltose. Maltotetraose (G4) was the smallest donor in transglycosylation reactions by AMTS. This is the first study that reports transglycosylation activity of AMTS on maltooligosaccharides. The results of this study suggest that high purity maltotriose can be produced by the hydrolytic action of AMTS on starch.

1. Introduction

Maltotriose has excellent properties, for example, mild sweetness, moisture preservation, low freezing-point depression, and structural relaxation reduction, which favors glassy states and prevents starch retrogradation.[1–3] Owing to these characteristics, maltotriose can be used in the production of desserts, baked goods, and as glucose replacement in intravenous feeding.[1,4] Researchers have recently reported that maltotriose is a nuclear localization signal for nanoparticles and proteins and that maltotriose-modified poly (propylene imine) dendrimers have physiological roles in cells.[5,6] Moreover, modified maltotrioses can be used as novel enzyme substrates[7,8] and as physiological activators.[9–11] However, due to its high price, utilization of maltotriose is limited.

Maltotriose can be obtained from the enzymatic hydrolysis of pullulan and starch and from maltose syrup purification.[1,4] High purity maltotriose can easily be produced by the hydrolytic action of pullulanase on pullulan;[4] however, pullulan is quite expensive. The other two production methods usually result in maltotriose of low purity. A low cost production of high purity maltotriose is of outmost importance for large scale applications. It has been reported that the starch hydrolysates derived from the enzymatic activity of glucan 1,4-alpha-maltotriohydrolase (AMTS, EC 3.2.1.116) consist predominantly of maltotriose.[12,13] Recent chromatographic separation developments provide a convenient method of producing pure maltotriose from low purity syrup at a relative low cost.[14,15] From a practical viewpoint, amylolysis by AMTS represents a potential method of producing maltotriose at low costs and for large scale applications. This study explored the production of maltotriose from starch using AMTS.

AMTS from *Natronococcus* sp. Ah-36 belongs to the family 13 of glycoside hydrolases (http://www.cazy.org/). AMTS successively removes maltotriose units from the non-reducing ends of polysaccharide chains, producing maltotriose as the main end product (http://www.brenda-enzymes.org). However, the mechanism of action of AMTS has not been elucidated.

In this study, a commercial AMTS from *Microbacterium imperiale* was purified and characterized. The objective of this study was to assess the hydrolytic mechanism of AMTS on corn starch (CS), corn amylose (CSAM), and corn amylopectin (CSAP). Additionally, we assessed the hydrolytic mechanism of AMTS on maltooligosaccharides, that is, maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7).

2. Results and discussion

2.1. Enzyme purification and characterization

To purify *M. imperial* AMTS, the commercial enzyme was fractionated using a HiTrap™ desalting column. The desalted

Abbreviations: AMTS, glucan 1,4-α-maltotriohydrolase; G1–G7, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose; DM, degree of starch hydrolyzed into maltotriose; PM, percentage of maltotriose in G1–G7; CS, corn starch; CSAM, corn amylose; CSAP, corn amylopectin; DP, degree of polymerization.

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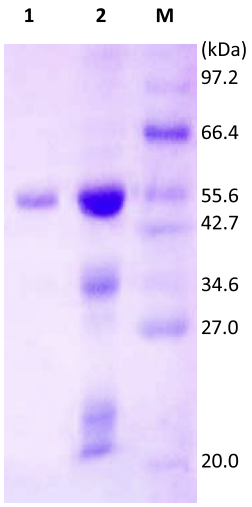

Figure 1. SDS–PAGE of AMTS in 10% polyacrylamide gel. Lane 1: purified AMTS; lane 2: commercial AMTS; lane M: molecular weight standards.

enzymatic fraction was applied to a cation-exchange chromatography column using HiTrap™ Q fast flow, yielding a single peak (AMTS) that corresponded to a single band in SDS-PAGE (Fig. 1). The optimum AMTS hydrolytic conditions consisted of a temperature of 50 °C and a pH of 6.5 (Fig. 2). These results are in accordance with those reported by other researchers.[12,13]

2.2. Maltotriose production from starch using AMTS

This study explored the production of maltotriose from inexpensive sources like CS. CSAM and CSAP were prepared to assess the AMTS hydrolytic mechanism. Figure 3 shows distinct hydrolytic patterns of AMTS on CS, CSAM, and CSAP as a function of time.

Figure 3 shows that the degree of starch hydrolyzed into maltotriose (DM) increased with hydrolysis. During the initial phase of hydrolysis, DM of CSAP was 2.3-fold higher than that of CSAM. However, these differences were not significant after 180 min of hydrolysis. Subsequently, DM of CSAM was higher than that of CS and CSAP. After extensive hydrolysis (24 h), DM was 79% for CSAM, 68% for CS, and 58% for CSAP. The relative high percentage of non-reducing ends and short chains in CSAP[16] may contribute to the higher releasing rates of maltotriose during the initial phase of hydrolysis. With extensive hydrolysis, the (1->6)-α-D-glucosidic linkages of CSAP may hinder AMTS activity, resulting in lower maltotriose releasing rates.

In the starch industry, the refining stage is of outmost concern because it is costly and time-consuming.[17] One considerable challenge is to separate sugars from low molecular weight sugars with similar chain lengths and physicochemical properties.[15] Therefore, our study evaluated the percentage of maltotriose in G1–G7 (PM) following hydrolysis by AMTS. In general, PM values of CS, CSAM, and CSAP were not decreased directly after hydrolysis. As a result of changing PM values, starch hydrolysis by AMTS appears to occur in three different stages (Fig. 3). In the first stage, PM values were high and subsequently decreased. In the second stage, PM values gradually decreased. In the last stage, PM values increased slightly and subsequently remained stable. At this inflection point, DM values were 50% for CSAP, 56% for CS, and 66% for CSAM. Low molecular weight dextrin molecules increased following starch hydrolysis.[18] AMTS activity on these dextrin molecules increased with increasing hydrolysis. The increased PM values during the last stage of hydrolysis might be attributed to the hydrolysis of low molecular dextrins.

Therefore, there are three different steps in starch hydrolysis by AMTS: in the first step, AMTS hydrolyzes starch molecules into low molecular dextrins with the accumulation of maltotriose; in the second step, AMTS simultaneously hydrolyzes the initial substrate and end products (i.e., low molecular dextrins); and in the final step, AMTS mainly hydrolyzes the remaining low molecular sugars. In addition, PM values of CSAP had a purity of >90% before reaching 10% DM, suggesting that pure maltotriose might be produced by a controlled partial hydrolysis of amylopectin. These observations reveal that starch structure and hydrolysis time determine the profiles of low molecular hydrolysates from starch by AMTS.

2.3. Hydrolysis of maltooligosaccharides (G2–G7)

Extensive hydrolysis of maltooligosaccharides by AMTS was monitored by HPAEC-PAD; the results are shown in Figure 4. G2

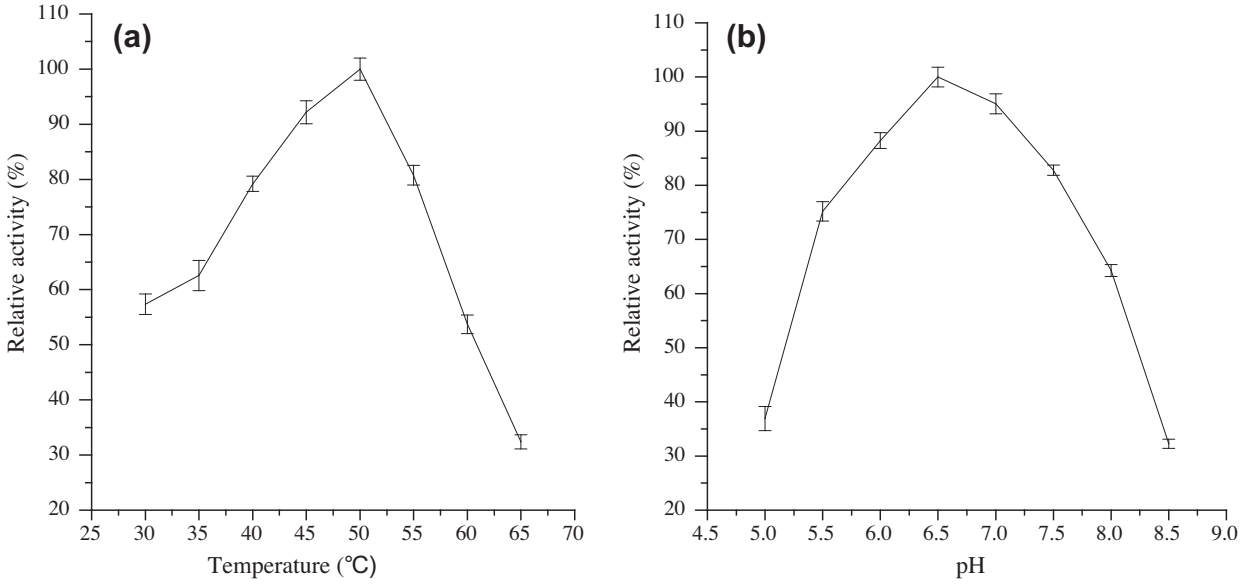

Figure 2. Optimum temperature and pH of AMTS. (a): Optimum temperature; (b): optimum pH.

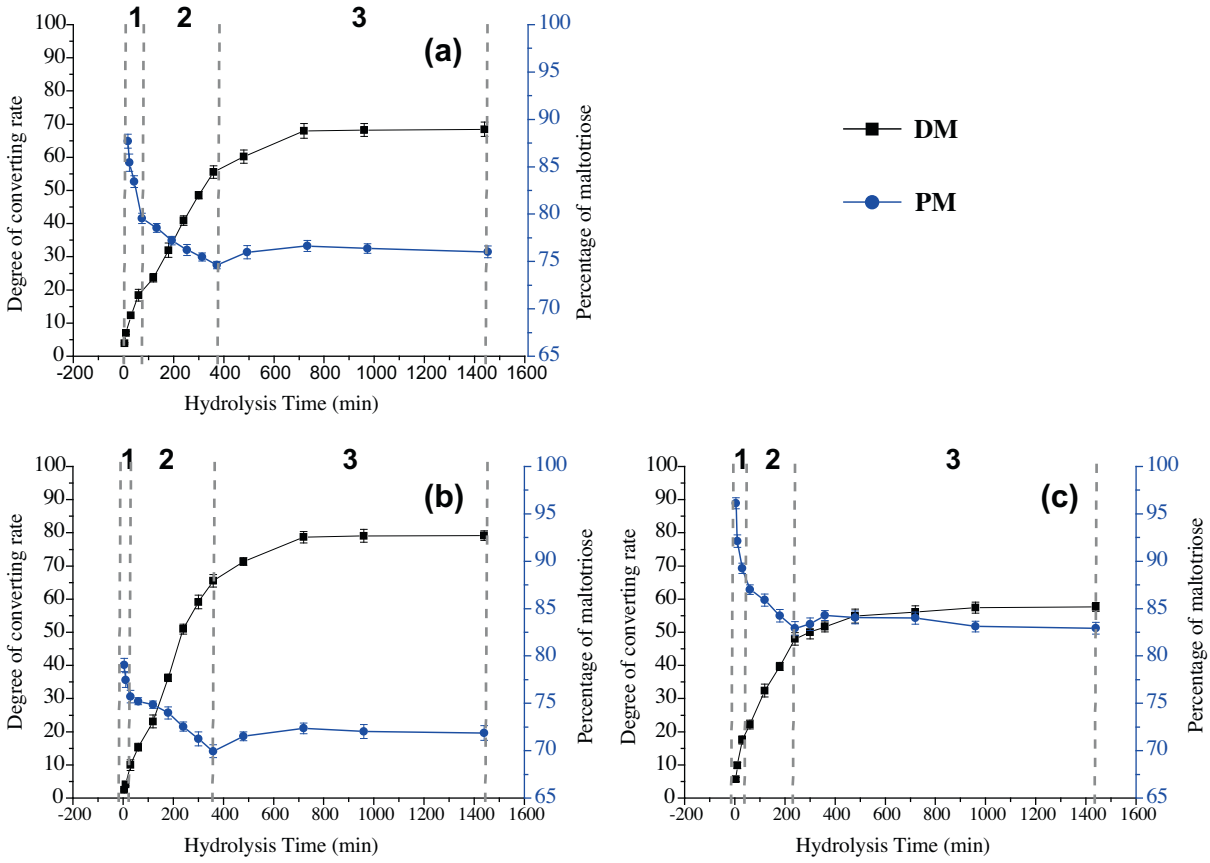

Figure 3. DM and PM values following starch hydrolysis by AMTS. (a): Corn starch; (b): amylose from corn starch; (c): amylopectin from corn starch. The numbers 1, 2, and 3 represent the hydrolysis stage 1, 2, and 3, respectively.

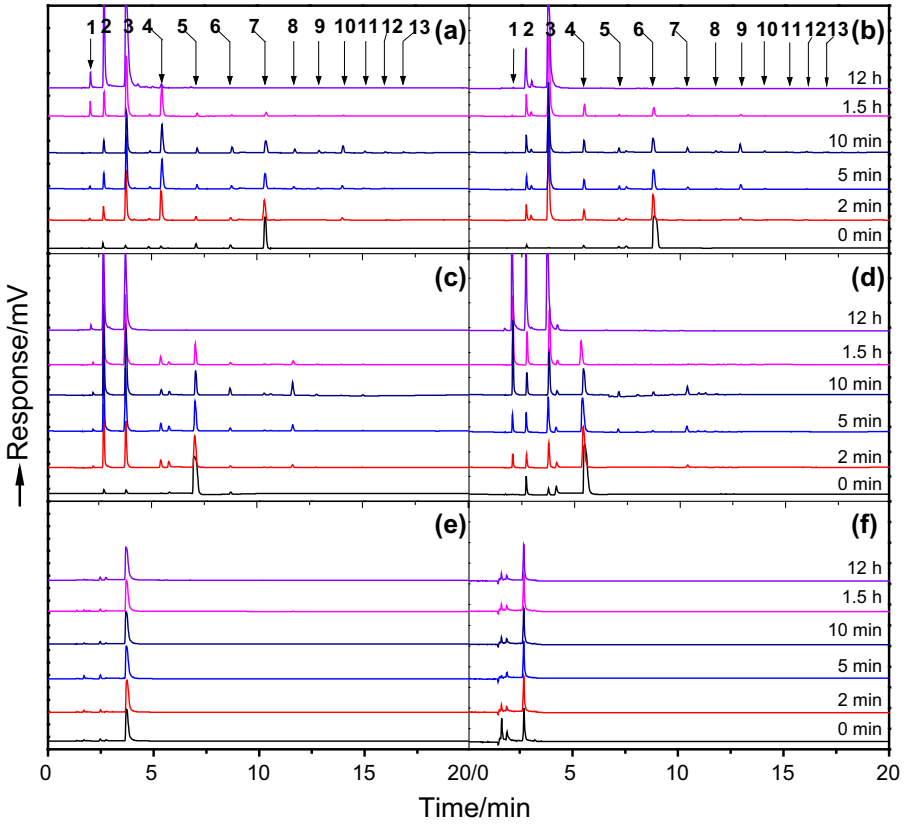

Figure 4. Maltooligosaccharides (G2–G7) hydrolyzed by AMTS. (a): Maltoheptaose (G7); (b): maltohexaose (G6); (c): maltopentaose (G5); (d): maltotetraose (G4); (e): maltotriose (G3); (f): maltose (G2). The numbers 1–13 represent sugars with a degree of polymerization (DP) 1–13.

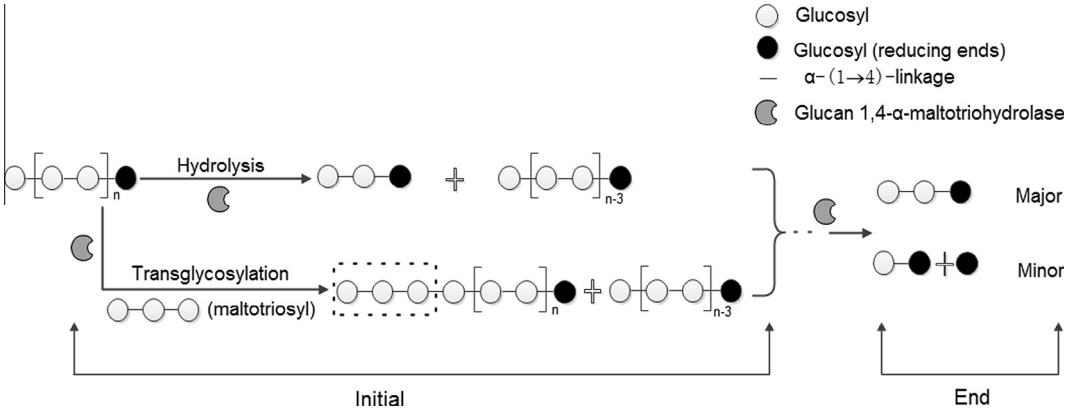

Figure 5. Proposed mechanism of *M. imperiale* glucan 1,4-α-maltotriohydrolase on maltooligosaccharides.

and G3 were relatively stable during hydrolysis; transglycosylation activity was observed during the hydrolysis of G4–G7. Therefore, the smallest donor in transglycosylation reactions by AMTS was G4. This is the first study that reports transglycosylation activity of AMTS. Similarly, α-amylases from *Byssochlamys fulva* and *Thermotoga matitima* have the capacity to perform transglycosylation reactions to their own hydrolysates.[19,20] After 12 h of incubation of G4–G7, different proportions of G1, G2, and G3 were produced. Hydrolysis rate increased with increasing substrate molecular weight as follows, G7 > G6 > G5 > G4 > G3 = G2 = 0.

During the initial phase of hydrolysis, the hydrolysis of G4 primarily resulted in G1 and G3 with a small number of G7 (Fig. 4). G5 hydrolysis mainly yielded G2 and G3, followed by G8; G6 hydrolysis mainly yielded G3 and a small amount of G9; and G7 yielded G3 and G4 and a small amount of G10. Apart from maltotriose, AMTS partially hydrolyzed maltooligosaccharides with DP_n ($n = 4$, 5, 6, and 7) into products with $DP_{n\pm3}$. The products apart from those with DP $n \pm 3$ obtained after 5 min or 10 min of hydrolysis (Fig. 4) might result from the hydrolysis of initial transglycosylated products by AMTS.

This is the first study that reports that maltotriosyl transfer occurs during the hydrolysis of maltooligosaccharides. To the best of our knowledge, glucosyl and maltosyl transferases have been previously reported; however, reports on spontaneous maltotriosyl transferases are scarce.[21,22] Therefore, the maltotriosyl transfer activity of AMTS can be used to produce special transglycosylated products.

Based on these results, the proposed mechanism of action of AMTS on maltooligosaccharides is showed in Figure 5. At the beginning, hydrolysis and maltotriosyl transfer reactions occur simultaneously. Following extensive hydrolysis, maltotriose is the major product with different proportions of glucose, maltose, and other by-products. This mechanism is important to understand the hydrolytic behavior of AMTS on CS. The different PM values from the hydrolysis of CS, CSAP, and CSAM could partially be attributed to the hydrolytic action of AMTS on different low molecular sugars released from starch.

3. Conclusions

This study described the production of maltotriose from starch hydrolysis by AMTS. Maltotriose production by AMTS depends on the specific starch structure (e.g., amylose versus amylopectin) and hydrolysis time. A specific transglycosylation activity by AMTS was elucidated. AMTS preferably transfers maltotriosyl units during the initial phase of hydrolysis. The smallest donor of transglycosylation reactions by AMTS was maltotetraose (G4).

4. Materials and methods

4.1. Materials

Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (G2–G7) were obtained from Sigma-Aldrich Trading Co., Ltd (Shanghai, China). Soluble starch (acid-hydrolyzed potato starch) was supplied by Sinapharm Chemical Reagent Co., Ltd (Shanghai, China). AMTS was purchased from Amano Enzyme Inc. (Nagoya, Japan).

4.2. Enzyme purification and characterization

AMTS was partially purified using the ÄKTA purifier™ 10 system (GE Healthcare, Uppsala, Sweden) equipped with a UV detector and a fraction collector. HiTrap™ desalting and HiTrap™ Q FF columns (GE Healthcare, Uppsala, Sweden) were used for purification. Fractions of high AMTS activity were pooled. Protein purity was assessed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was determined by the Coomassie brilliant blue method with bovine serum albumin as the standard.[23]

To identify the optimum pH of AMTS, enzymatic assays were performed in 1% soluble starch dissolved in 0.1 M phosphate buffer at different pH values (pH 5.0–8.5). The optimum temperature of AMTS was determined by incubating AMTS in 1% soluble starch at different temperatures (30–65 °C). Samples were periodically removed; the ratio (in percentage) between the enzyme activity at a certain pH or temperature and the corresponding optimum activity was calculated.

AMTS activity was determined by quantifying the amount of reducing sugars[24] released from 1% soluble starch in 0.1 M phosphate buffer (pH 6.5) at 50 °C. Maltotriose (0–1 μmol/mL) was used as a standard. The results were expressed as μmol of maltotriose equivalents released per min. One enzymatic unit (U) was defined as the amount of enzyme required to release 1.0 μmol of maltotriose equivalents per min.

4.3. Enzymatic hydrolysis of starch into maltotriose

4.3.1. CS fractionation

CSAM and CSAP were separated from CS according to the method reported by Takeda et al.[25,26] CS was defatted three times with 90% dimethyl sulfoxide and precipitated with 6 volumes of ethanol. The resulting residue was vacuum-dried at 40 °C for 24 h. The dried residue was re-dissolved and precipitated with *n*-butyl alcohol at 4 °C for 24 h. Following centrifugation, CSAM and CSAP

were separated from each other. Crude CSAM was repeatedly precipitated in saturated *n*-butyl alcohol solution until the purity was >98%. For CSAP preparation, the supernatant was further precipitated with *n*-butyl alcohol to remove amylose. The precipitate was discarded. Ethanol was added to the supernatant and stored overnight at 4 °C. This procedure was repeated several times. Subsequently, CSAM and CSAP were washed in ethanol and vacuum dried for purification purposes.

4.3.2. Preparation of starch dispersions

Aqueous starch dispersions (1%, w/v) were prepared according to the protocol reported by Yoo and Jane.[27] CS, CSAM, and CSAP were dispersed in DMSO (95%, w/v) under constant stirring at 100 °C for 60 min. The dispersions were further stirred for 24 h at 30 °C. Ethanol was added to precipitate starch, which was suspended in acetone. Following centrifugation, the starch pellet was vacuum dried, dispersed in boiling 0.1 M phosphate buffer (pH 6.5), and stirred for 60 min.

4.3.3. Product profiles analysis by HPLC

CS, CSAM, and CSAP dispersions (1% w/v, pH 6.5, 39 mL) were equilibrated at 50 °C; 1 mL of AMTS (200 U/mL) was added. Samples were periodically removed after 5, 10, 30, 60, 120, 180, 240, 300, 360, 480, 720, 960, and 1,440 min of incubation. The mixtures were precipitated with ethanol (1:1, v/v), filtered, and injected into a high performance liquid chromatography (HPLC) system (Chromaster, Hitachi High-Tech Corp., Tokyo, Japan) with a 5425 refractive index detector. A Hypersil APS2 column (4.6 mm × 250 mm, 5 μm), maintained at 40 °C, was used. The mobile phase consisted of acetonitrile (72%, v/v) at 1 mL/min. Quantification of maltotriose was performed by comparing the peak areas of unknown samples with those of maltotriose standards. DM was calculated using the following equation,

$$\mathrm{DM} = \frac{\text{Maltotriose content quantified by HPLC (mg)}}{\text{Search content (mg, dry basis)}} \times 100$$

From the peak areas corresponding to the digestion of different substrates by AMTS, PM was calculated using the following equation,

$$\mathrm{PM} = \frac{\text{Peak areas of maltotriose}}{\text{Total peak areas of low molecular sugars (G1}-\text{G7)}} \times 100.$$

4.4. Hydrolysis of maltooligosaccharides (G2–G7)

The hydrolytic activity of AMTS was assessed using maltooligosaccharides (G2–G7). Maltooligosaccharides were dissolved in 0.1 M phosphate buffer (1%, pH 6.5) and mixed with AMTS (100 μl, 10 U). The hydrolysates obtained after 2 min, 5 min, 10 min, 1.5 h and 12 h were analyzed by high performance anion-exchange chromatography with a pulsed amperometric detection system (HPAEC-PAD; Dionex DX 500, Sunnyvale, CA, USA) coupled with a GP40 gradient pump and an electrochemical detector. Bound material was eluted with 0.15 M NaOH and a sodium gradient at 1 mL/min.[28,29]

4.5. Statistical analyses

Data were analyzed using PASW Statistics 18 (SPSS Inc., Chicago, USA). All results were reported as mean ± SD by single-tree analysis in triplicate.

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References

1. Singh, R. S.; Saini, G. K.; Kennedy, J. F. *Carbohydr. Polym.* **2010**, *80*, 401–407.
2. Syamaladevi, R. M.; Barbosa-Cánovas, G. V.; Schmidt, S. J.; Sablani, S. S. *Carbohydr. Polym.* **2012**, *88*, 223–231.
3. Shirke, S.; Ludescher, R. D. *Carbohydr. Res.* **2005**, *340*, 2654–2660.
4. Wu, S.; Chen, H.; Tong, Q.; Xu, X.; Jin, Z. *Eur. Food Res. Technol.* **2009**, *229*, 821–824.
5. Akita, H.; Masuda, T.; Nishio, T.; Niikura, K.; Ijiro, K.; Harashima, H. *Mol. Pharm.* **2011**, *8*, 1436–1442.
6. Drzewinska, J.; Appelhans, D.; Voit, B.; Bryszewska, M.; Klajnert, B. *Biochem. Biophys. Res. Commun.* **2012**, *427*, 197–201.
7. Damager, I.; Numao, S.; Chen, H.; Brayer, G. D.; Withers, S. G. *Carbohydr. Res.* **2004**, *399*, 1727–1737.
8. Liu, L.; Pohl, N. L. B. *Carbohydr. Res.* **2013**, *369*, 14–24.
9. Ferrer, M.; Perez, G.; Plou, F. J.; Castell, J. V.; Ballesteros, A. *Biotechnol. Appl. Biochem.* **2005**, *42*, 35–39.
10. Vismara, E.; Coletti, A.; Valerio, A.; Naggi, A.; Urso, E.; Torri, G. *Molecules* **2012**, *17*, 9912–9930.
11. Thiebault, N.; Lesur, D.; Godé, P.; Moreau, V.; Djedaïni-Pilard, F. *Carbohydr. Res.* **2008**, *343*, 2719–2728.
12. Yang, C.-H.; Liu, W.-H. *Enzyme Microb. Technol.* **2004**, *35*, 254–260.
13. Takasaki, Y.; Kitajima, M.; Tsuruta, T.; Nonoguchi, M.; Hayashi, S.; Imada, K. *Agric. Biol. Chem. Tokyo* **1991**, *55*, 687–692.
14. Lee, J.; Kwon, T.; Moon, I. *Biotechnol. Bioprocess Eng.* **2003**, *8*, 47–53.
15. Xie, Y.; Chin, C. Y.; Phelps, D. S. C.; Lee, C.-H.; Lee, K. B.; Mun, S.; Wang, N.-H. L. *Ind. Eng. Chem. Res.* **2005**, *44*, 9904–9920.
16. Tester, R. F.; Karkalas, J.; Qi, X. *J. Cereal Sci.* **2004**, *39*, 151–165.
17. Lee, J. W.; Kwon, T. O.; Moon, I. S. *Biotechnol. Bioprocess Eng.* **2003**, *8*, 47–53.
18. Baks, T.; Bruins, M. E.; Matser, A. M.; Janssen, A. E. M.; Boom, R. M. *J. Agric. Food Chem.* **2007**, *56*, 488–495.
19. Doyle, E. M.; Noone, A. M.; Kelly, C. T.; Quigley, T. A.; Fogarty, W. M. *Enzyme Microb. Technol.* **1998**, *22*, 612–616.
20. Park, H.; Kim, J.; Choi, K.-H.; Hwang, S.; Yang, S.-J.; Baek, N.-I.; Cha, J. *J. Agric. Food Chem.* **2012**, *60*, 8183–8189.
21. Toshiyuki, S.; Nobukazu, H.; Jun, S.; Satoru, U.; Yuki, H.; Kuniki, K.; Kohtato, K. J. *Mol. Catal. B: Enzym.* **2012**, *80*, 20–27.
22. Moreno, A.; Damian-Almazo, J. Y.; Miranda, A.; Saab-Rincon, G.; Gonzalez, F.; Lopez-Munguia, A. *Enzyme Microb. Technol.* **2010**, *46*, 331–337.
23. Sedmak, J. J.; Grossberg, S. E. *Anal. Biochem.* **1977**, *79*, 544–552.
24. Somogyi, M. *J. Biol. Chem.* **1945**, *160*, 61–68.
25. Takeda, Y.; Hizukuri, S.; Juliano, B. O. *Carbohydr. Res.* **1986**, *148*, 299–308.
26. Thitipraphunkul, K.; Uttapap, D.; Piyachomkwan, K.; Takeda, Y. *Carbohydr. Polym.* **2003**, *54*, 489–498.
27. Yoo, S.-H.; Jane, J.-L. *Carbohydr. Polym.* **2002**, *49*, 307–314.
28. Cataldi, T. R. I.; Campa, C.; De Benedetto, G. E. *Fresenius J. Anal. Chem.* **2000**, *368*, 739–758.
29. Hattori, T.; Kato, Y.; Uno, S.; Usui, T. *Carbohydr. Res.* **2013**, *366*, 6–16.