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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Preferential and rapid degradation of raw rice starch by an α-amylase of glycoside hydrolase subfamily GH13_37

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Abstract The α-amylase (AmyP) from a marine metagenomic library belongs to the recently classified glycoside hydrolase subfamily GH13_37. The degradation abilities of AmyP on a broad range of raw starch granules were examined at 40 °C and pH 7.5. It was found that AmyP is a raw starch-degrading enzyme, exhibiting a unique and remarkable ability to preferentially and very rapidly digest raw rice starch. The specific activity of raw rice starch was reached 118.5±0.6 Umg^{-1}, which was much higher than that of other raw starches. The final hydrolysis degrees were obtained in 4 h for 1 % raw rice starch and 1 h for 8 % concentration, indicating a very rapid speed of hydrolysis. The presence of a starch residue resistant was the main limiting factor for complete hydrolysis, although end product inhibition also existed, especially at high starch concentrations. AmyP randomly attacks unique or susceptible sites on raw rice starch granules, and releases glucose, maltose, and maltotriose as end products. This is the first biochemical characterization of the raw starch-degrading ability of an α-amylase of family GH13_37. The specific ability towards raw rice starch has never been described before, and this makes AmyP a promising candidate for use as a novel enzyme in rice starch processing.

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Introduction

Starch has been extensively studied for food application as well as other applications such as pharmaceutics, papers, adhesives, packaging, and biofuels (Jobling 2004; Qin et al. 2011). Native raw starch is biosynthesized as densely packed semicrystalline granules. Most of these applications require disruption of starch granules through high temperature, acid, alkaline, or enzyme (Robertson et al. 2006; Tawil et al. 2011). Enzyme hydrolysis of raw starch is energy efficient and environmentally friendly. However, many properties of starch granules, including type of crystal polymorph, surface microstructure, granular architecture, and granular size, greatly limit the susceptibility of starch granules to enzymatic degradation (Tester et al. 2006). Therefore, raw starch-degrading enzymes have drawn the attention of researchers not only due to the value to "green" manufacturing but also to understand the structure of starch granules.

α-Amylases (E.C 3.2.1.1) are widely occurring enzymes, which randomly hydrolyze α-1,4-glycosidic linkages in starch and related carbohydrates. Based on amino acid sequence, most α-amylases are classified in glycoside hydrolase (GH) family 13. They are among the most important raw starch-degrading enzymes, although only a limited number of α-amylases seem to be able to degrade raw starch (Robertson et al. 2006; Sun et al. 2010). The vast majority of raw starch-degrading α-amylases are produced by terrestrial organisms. So far, only two α-amylases from marine bacteria *Bacillus* sp. ALSHL3 (Vidilaseris et al. 2009) and *Bacillus aquimaris* MKSC 6.2 (Puspasari et al. 2011) have been

reported to be able to digest raw starch. Recently, we isolated a novel α-amylase (AmyP) from a marine metagenomic library (Liu et al. 2012). The discovery led to the identification of a new subfamily GH13_37, which has been regarded as an independent clade of ancestral marine bacterial α-amylases on the basis of sequence analysis. The members of GH13_37 show extremely low (no more than 20 %) sequence similarity to other known α-amylases and possess more aromatic residues within a catalytic domain, implying that the members may retain more primordial sequence features of the ancestor. At the time of writing, the subfamily GH13_37 contained 16 proteins [see the Carbohydrate-Active Enzyme (CAZy) database at www.cazy.org] (Cantarel et al. 2009). It is interesting to test whether these marine bacterial α-amylases have any exclusive features that are not present in other known α-amylases. Therefore, a broad range of substrate utilization capabilities of AmyP were investigated. The results reveal that AmyP has a remarkable raw starch-degrading activity, especially towards raw rice starch, and in this respect, it appears a preferential and very rapid hydrolytic ability that has not been described before.

Materials and methods

Gene expression and enzyme purification

Escherichia coli BL21 (DE3) cells harboring pET32a-*AmyP* (Liu et al. 2012) were used for enzyme production. The cells were grown with agitation at 37 °C to OD_{600} of 0.7, induced with isopropyl-β-D-thiogalactopyranoside, and further incubated at 16 °C for 12 h to induce AmyP. The cells were harvested and lysed by ultrasonication. AmyP protein was purified with one-step purification procedure using Ni^{2+} affinity chromatography (Invitrogen). The nucleotide sequence was deposited in the GenBank database with accession number of HM572234.

Enzyme and protein assays

Raw starch-degrading activity was determined by measuring the amount of reducing sugar using 3′5-dinitrosalicylic acid (DNS) as described by Miller (1959). A standard reaction was determined at 40 °C for 5 min. The reaction mixture contained 100 μl appropriately diluted purified AmyP and 500 μl 4 % raw rice starch (or other raw starches as specified in the text) in 100 mM sodium/potassium phosphate buffer (pH 7.5). All starches were washed five times with MilliQ water and dried in a desiccator before use. When hydrolysis time was longer than 10 min, 0.2 % toluene was added to the reaction mixture to prevent microbial contamination (Hamilton et al. 1998). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar as glucose standard from the substrate per minute (Mitsuiki et al. 2005). Protein concentration was determined by using the Bradford method with bovine serum albumin as a standard.

Effect of pH and temperature on raw starch-degrading activity

The effect of the pH value on raw starch-degrading activity was measured at 40 °C in 100 mM sodium/potassium phosphate (pH 5.0 to 8.0) and 100 mM glycine–NaOH (pH 8.5 to 9.0), adjusted at this temperature to various pH values, using the standard assay described above. The effect of the temperature on the activity was determined at pH 7.5 using the standard assay reaction mixtures incubated at temperatures ranging from 0 to 70 °C. The thermostability of AmyP was determined through preincubation of the enzyme under the optimum conditions for various periods of time followed by determination of the residual activity with the standard assay.

Kinetics of amylolysis at different starch concentrations

Four starch concentrations were used: 1, 4, 8, and 12 %, respectively. Fifty units of AmyP was added to the starch suspensions and the final volume adjusted to 6 ml with buffer. Thus, 0.83, 0.17, 0.10, and 0.07 U per mg dry starch were used for 1, 4, 8, and 12 % starch, respectively. The suspensions were shaken continuously in a water bath at 100 rpm, and 0.6-ml aliquots were withdrawn at different time intervals until 4 h. For each aliquot, the reaction was stopped by cooling in an ice-cold 0.3 M Na_2CO_3 stop solution (Tahir et al. 2010) and then centrifuged at 8,000×*g* at 4 °C for 5 min. The reducing sugars in the supernatant were determined by the DNS method. The degree of degradation was expressed as the ratio of soluble sugars from starch hydrolysis to the initial mass of starch (Iefuji et al. 1996).

High-performance liquid chromatography of hydrolytic products

The hydrolytic products of AmyP from raw rice starch were determined by high-performance liquid chromatography (HPLC; Agilent 1260 system) using a Carbomix Ca-NP (250×4.5 mm) column (Sepax Technologies, Inc., USA) and a refractive index detector. MilliQ water was used as the mobile phase with a flow rate of 0.1 ml min^{-1} at 80 °C. Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), and maltopentaose (G5) were used as standards.

Scanning electron microscopy

A mixture containing 1 % raw rice starch and 50 U of AmyP was incubated at 40 °C for 30 min. The mixture was centrifuged at 8,000×*g*, and the pellet was washed three times with pure ethanol followed by drying at 35 °C. Starch granules were attached to scanning electron microscopy (SEM) holder and then coated with Pt using Ion Sputter E-1010 at 5.0 kV and 20 mA for 40 s. Samples were inserted into specimen chamber SEM (HITACHI S-4800), and the pictures were taken.

Determination of the limiting factors for a complete hydrolysis

To check the potential inhibition of AmyP by end products or resistant residual structure, 1 and 8 % raw rice starches were hydrolyzed adequately for 2 and 1 h at 40 °C, respectively. The residues were washed three times with buffer by centrifugation at 8,000×*g*. Then, a new fresh AmyP solution (50 U) was added and a secondary hydrolysis was performed under the same condition. Each sample was adjusted to a final volume of 0.6 ml. Furthermore, the degree of inhibition on AmyP activity by end products was further determined. After 10 min of incubation at 80 °C to inactivate the enzyme, the first hydrolysate was centrifuged to obtain the end products in the supernatant. The secondary hydrolysis was conducted as described above after adding the end products. The degree of inhibition on AmyP was quantified by measuring the reducing sugars released during the hydrolysis. At least five measurements were made for each condition, and the data given are an average of these results.

Results

Effect of pH and temperature on the raw starch-degrading ability of AmyP

Expression of AmyP at low temperature (16 °C) resulted in the accumulation of majority of the recombinant protein in the soluble fraction. The maximum expression level of AmyP in *E. coli* reached 36±4.4 mg l^{-1} after 12 h induction. Recombinant AmyP fused with a C-terminal His-tag was easily purified to homogeneity with more than 80 % recovery over the Ni^{2+} affinity chromatography (Fig. 1).

With an assay using raw rice starch as the substrate, AmyP displayed the maximum activity at pH 7.5 and 40 °C (Fig. 2a, b). Between pH 6.5 and 8.0 there are still 79 % of activity remaining, while no activity was detected at pH value lower than 5.0 or higher than 9.0. AmyP was very sensitive to temperature inactivation at pH 7.5, losing more than 65 % of the activity at 30 or 50 °C. But the enzyme showed good

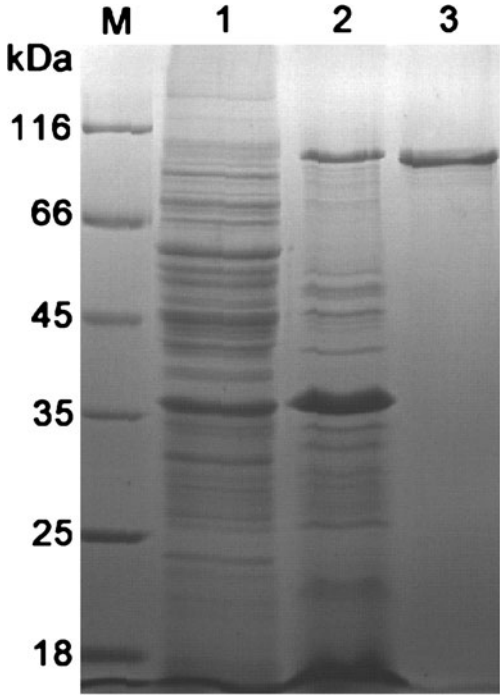

Fig. 1 SDS-PAGE analysis of recombinant AmyP. *Lane M*, size marker proteins; *lane 1*, cell extract of *E. coli* harboring pET32a; *lane 2*, the supernatant of cell extract of *E. coli* harboring pET32a-*AmyP*; *lane 3*, the purified AmyP protein

stability under the optimum pH and temperature (Fig. 2c), and maintained 60 % of the original activity after 4 h of incubation. In addition, the maximum activity of AmyP towards soluble starch was displayed at 50 °C and pH 6.5 (Liu et al. 2012). A similar shift in optimum pH and temperature was also seen in three glucoamylases from *Corticium rolfsii* (Nagasaka et al. 1998).

Hydrolysis of various raw starches by AmyP

Native starch granules exhibit two main allomorphic types (A or B type). The A type mainly occurs in cereal starches and the B type in tubers and amylose-rich starches. Another C type, a mixed A and B type, is characteristic of most legume starches (Buléon et al. 1998). Various types of raw starches were examined as substrates to compare the raw starch-degrading ability of AmyP (Table 1). It was observed that the enzyme could hydrolyze a broad range of raw starch granules in very short hydrolysis time (5 min) at 40 °C. Remarkably, AmyP exhibited a high specific activity towards raw rice starch (118.5±0.6 U mg^{-1}), which was much higher than that of other raw starches.

Hydrolysis of raw rice starch at different concentrations

With a constant enzyme dose, raw rice starch was hydrolyzed with increasing concentrations for time periods

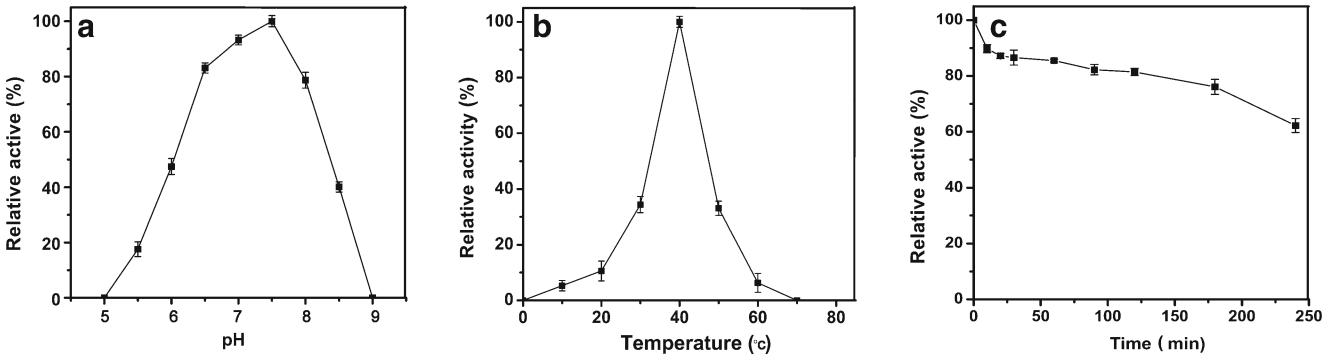

Fig. 2 Effects of pH and temperature on the activity of AmyP towards raw rice starch. **a** pH; **b** temperature; **c** heat stability at 40 °C, pH 7.5

ranging from 10 min to 4 h (Fig. 3a). Its hydrolysis characteristics were compared to that of raw wheat starch (Fig. 3b), which also is a cereal starch and has been widely used as a common substrate in raw starch hydrolysis studies. No matter at what starch concentration tested, AmyP is much more effective on raw rice starch than wheat starch. In both cases, the release of reducing sugar increased significantly with increasing starch concentration from 1 to 8 %, while the enzyme activity was slightly decreased in the presence of 12 % starch. Thus it appears that the enzymatic efficiency is highly dependent on the starch concentration, which is in good agreement with the previous study of classical porcine pancreatic α-amylase (Kong et al. 2003).

The two hydrolysis curves have a classical two-phase shape with an initial logarithmic phase followed by an asymptotic phase. But the initial phase of rice starch hydrolysis was unusually short, less than 10 min for 1 % and 30 min for 4 and 8 % starch suspensions, respectively. A remarkably different initial phase was noticed in the wheat starch curve, which was extended to 2 h for 1 and 4 % and 3 h for 8 %, respectively. The results suggested that AmyP could hydrolyze raw rice starch at a very high speed.

Action pattern of AmyP on raw rice starch

Scanning electron microscopy was used to visualize raw starch samples hydrolyzed by AmyP. The surface of untreated rice starch granules was smooth and angular (Fig. 4a). The enzyme action resulted in large and deep holes on the surface of rice starch granules, and the edge of rice starch granules became smooth (Fig. 4b), indicating that the modes of attack included both exo-corrosion and endo-corrosion. However, the high-efficiency degradation did not occur homogeneously and a small quantity of rice starch granules displayed small and shallow holes (data not shown). The unhomogeneous attack suggested that the action modality of AmyP was a random attack at unique or susceptible sites of raw rice starch. The modes of attack on raw rice starch granules were compared with that of raw wheat starch granules. Untreated wheat starch granules were shown in Fig. 4c. Although hydrolyzed raw wheat starch granules displayed large and deep holes on surface, the number of holes is very small (Fig. 4d). Due to poor catalytic activity of AmyP towards raw wheat starch, the eroded wheat starch granules accounted for only a small proportion in the total granules compared to hydrolyzed rice starch granules.

To clarify the hydrolysis pattern, 8 % raw rice starch was hydrolyzed at various time durations, and the soluble products were analyzed by HPLC (Table 2). G1, G2, G3, G4, G5, and larger maltodextrins were the soluble oligosaccharides at the initial stage of the hydrolysis. The composition changed significantly between 10 and 60 min. G1, G2, and G3 became the final oligosaccharides. Such a final composition is in agreement with that of classical saccharifying-type α-amylase, which produces predominantly G1, G2, or G3 as end products (Liu et al. 2012; Ohdan et al. 1999). However, the observations were slightly different from previous studies on soluble starch where AmyP action resulted in G1, G2, and G3 as final oligosaccharides within 5 min of hydrolysis (Liu et al. 2012), which was not surprising when considering the lower accessibility and mobility of raw starch.

Table 1 Hydrolysis activities of AmyP on various raw starches

Starch source	Activity (U mg^{-1})	Relative activity (%)
A type		
Rice	118.5±0.6	100
Wheat	7.2±0.2	6.1
B type		
Potato	12.3±0.58	10.4
C type		
Mung	17.9±1.2	15.1
Pea	8.7±0.3	7.3

Limiting factors for complete hydrolysis

The very rapid and short hydrolysis aroused great interest in the analysis of limiting factors in the enzymatic hydrolysis. Figure 2c shows the stability of AmyP under the buffer and

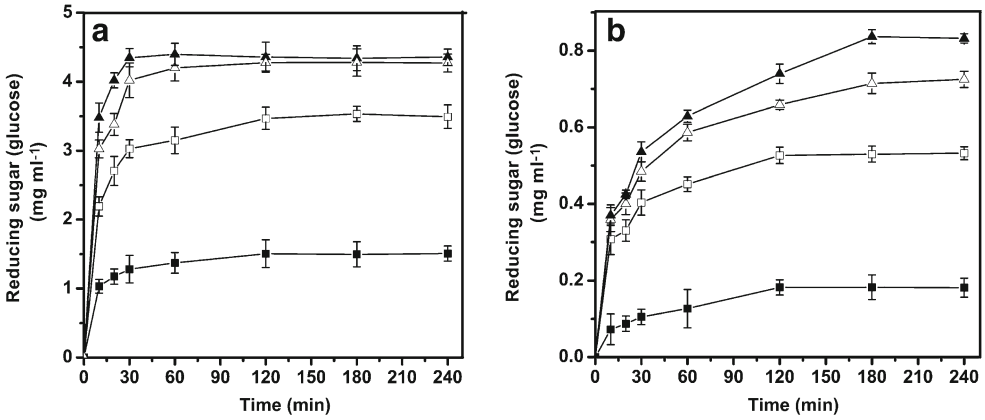

Fig. 3 Effect of raw starch concentration on AmyP hydrolysis kinetics. **a** Rice and **b** wheat (*filled square*—1 %, *empty square*—4 %, *filled triangle*—8 %, and *empty triangle*—12 %)

temperature conditions as used for hydrolysis. Under these conditions, 85 and 60 % of the original activity could be maintained after incubation for 1 and 4 h, respectively. Therefore, the effective action of AmyP on 8 % raw rice starch for only 1 h is not limited by a decrease in the activity of the enzyme.

A 2-h hydrolysis of 1 % raw rice starch was able to produce 1.5±0.2 mg ml^{-1} reducing sugar. However, very little reducing sugar was determined after washing the residue and adding a fresh dosage of AmyP, which meant that the addition of fresh enzyme did not lead to further high-efficiency hydrolysis, most likely because of a very resistant structure of the remaining starch residue. Furthermore, the results also indicated that no obvious inhibition by the end products was occurring when AmyP acted on 1 % raw starch.

For 8 % raw rice starch, 4.4±0.2 mg ml^{-1} reducing sugar was determined after 1 h of hydrolysis, while 1.3±0.1 mg ml^{-1} reducing sugar was observed in the secondary hydrolysis under the same conditions. It was suggested that both the starch residue resistant and the end products may play inhibitory roles in the hydrolysis of high starch concentrations. To further check the product inhibition, the inhibition effects of the end products released during the hydrolysis of 8 % raw rice starch were determined. No release of reducing sugar was detectable after adding the end products.

Discussion

Although raw starch-degrading α-amylases isolated from many different terrestrial sources were well studied (Sun et al. 2010), production of raw starch-degrading α-amylases by marine organisms is poorly documented, perhaps because of the conventional view that no starch plant grows

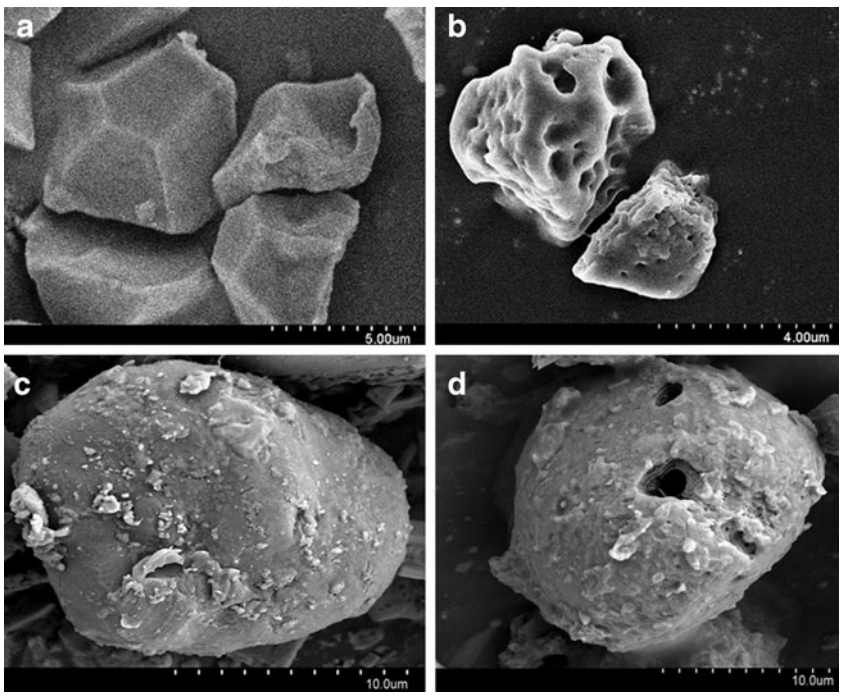

Fig. 4 Scanning electron microscopy of untreated (**a**, **c**) and treated (**b**, **d**) raw starch granules by AmyP. Starch granules of rice (**a**) and wheat (**c**)

Table 2 Composition in the soluble fraction during hydrolysis of 8 % raw rice starch by AmyP

Time (min)	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)	Maltodextrins (%)
10	13.8	35.9	25.7	5.0	8.7	10.8
20	15.3	33.7	43.6	1.6	2.5	3.3
30	17.2	32.3	48.6	0.5	1.3	0.0
60	17.4	33.4	49.2	0.0	0.0	0.0

in dark and cold deep sea. AmyP, derived from a marine metagenomic library of bottom sediment microflora, belongs to the recently classified subfamily GH13_37. By using different polysaccharides from marine organisms as hydrolytic substrates, we anticipated that this attempt would extend the range of display applications of α-amylases. The polysaccharides tested included fucoidan, laminarin, carrageenan, alginic acid, and chitin, and the hydrolysis products were assayed directly by HPLC. Our results show none of these polysaccharides could serve as substrates for AmyP. Therefore, it is somewhat surprising that AmyP is efficient in hydrolyzing all raw starches tested from terrestrial plants.

The raw starch-degrading AmyP shows two properties that are quite different from those of any other previously isolated raw starch-degrading α-amylases. First, it preferentially hydrolyzes raw rice starch because the specific activity of raw rice starch is much higher than that of other raw starches (Table 1). Rice starch and wheat starch are same for the A type, yet the specific activities can vary as much as 16 times depending on other attributes of enzymatic reactivity. In comparison with rice starch, the differences among other starches tested were not very significant. The data suggested that the preference was not dependent on allomorphic types of raw starches. Various α-amylases capable of degrading raw rice starch were summarized in Table 3. Although different starch concentration and enzyme amount were used for every particular α-amylase, it can be seen that most of the α-amylases have stronger digesting abilities towards wheat starch and corn starch than that of rice starch. The α-amylase Amy II from earthworm *Eisenia foetida* showed a similar preference towards raw rice starch (Ueda et al. 2008). However, compared with the high specific activity of AmyP for raw rice starch (118.5±0.6 $U mg^{-1}$), the specific activity of Amy II was only 18.7 $U mg^{-1}$. Secondly, AmyP is capable of digesting raw rice starch with a very rapid speed of hydrolysis. The hydrolysis occurred during the early 2 h for 1 % concentration and 1 h for 8 % (Fig. 3a). The most efficient hydrolysis time was less than 30 min. As listed in Table 3, the other α-amylases require at least 24 h to complete the hydrolysis. To our knowledge, the very rapid speed of hydrolysis was only observed in α-amylase from *Anoxybacillus flavothermus*

Table 3 Ability of AmyP and other α-amylases to digest raw starches

Origins	Degree of degradation (%)			Enzyme amount (U mg^{-1} starch) and starch concentration (%)	Incubation time	Incubation temperature (°C)	Incubation pH	Reference
	Rice	Wheat	Corn					
Bacteria								
Unknown marine bacterium, AmyP	22.6 (19.2)	2.7 (1.6)	4.0 (3.6)	0.83, 1	4 h (30 min)	40	7.5	Present work
Thermobifida fusca NTU22	17.0	ND	14.0	0.02, 5	24 h	50	7.0	Yang and Liu (2004)
Clostridium butyricum T-7	93	100	100	0.14, 1	72 h	37	6.0	Tanaka et al. (1987)
Bacillus sp. IMD 434	28	18	32	1, 1	24 h	40	6.0	Hamilton et al. (1998)
Bacillus amyloliquefaciens	26	17.4	26	5[a]	24 h	55	5.5	Demirkan et al. (2005)
Bacillus aquimaris MKSC 6.2	2	ND	23	0.11, 1	24 h	37	6.5	Puspasari et al. (2011)
Bacillus stearothermophilus NCA 26	63	ND	70	0.025, 0.4	50 h	40	7.0	Dettori-Campus et al. (1992)
Fungi and yeast								
Cryptococcus sp. S-2, AMY-CS2	28.5	42.0	46.0	0.03, 1	24 h	30	6.0	Iefuji et al. (1996)
Aspergillus oryzae, TAA	1.0	4.0	2.0	0.03, 1	48 h	30	5.0	Iefuji et al. (1996)
Animals								
E. foetida, Amy II	100.0	47.5	2.8	0.4[a]	96 h	37	5.5	Ueda et al. (2008)
Porcine pancreas, PPA	36	52.5	55	0.03, 1	24 h	30	7.0	Iefuji et al. (1996)

ND not determined

[a] No enzyme amount data

(Tawil et al. 2012). Its hydrolysis curve of raw corn starch also had a rapid and short initial phase, which did not exceed 2–3 h for 5 % concentration. There is no report of the α-amylase from *A. flavothermus* capable of preferentially hydrolyzing any raw starch. It is widely believed that the initial speed of hydrolysis was significantly correlated with the surface microstructure of raw starch, which was known as the "surface depressions" model (Juszczak et al. 2003; Robertson et al. 2006; Tahir et al. 2010). The hydrolyzed rice starch granules possessed much more holes on surface than that of wheat starch granules (Fig. 4). The holes of various sizes were observed in almost all hydrolyzed rice starch granules, while the holes were only found in a small proportion of hydrolyzed wheat starch granules. From this result, it is suggested that the amount of amylase-digestible regions in raw rice starch granules was greater than in raw wheat starch granules. Therefore, we hypothesize that the initially rapid speed of hydrolysis of raw rice starch is attributed to a specific recognition between AmyP and the surface microstructure of raw rice starch. In addition, the preferential and rapid degradation ability of AmyP opens a potential way not only for an enzymatic process for preparing high processed rice starch but also for the design of new industrial processing.

The process of raw starch digestion by α-amylase is complex. Previous reports have shown that the efficiency of digestion depended on many factors, such as enzyme activity, enzyme stability, enzyme inhibition by G1 and G2, and the botanic origin of starch granule (Robertson et al. 2006; Tawil et al. 2011; Tester et al. 2006). Knowledge about limiting factors is important for a better understanding of enzyme characterization. Our data suggested that the hydrolysis end of AmyP was chiefly due to the starch residue resistant, whereas the effect of the product inhibition did exist, especially at high starch concentrations. The starch residue resistant was mainly involved in the morphology and the surface microstructure of starch granules and the crystalline structure. It is well-known that the allomorphic B type structure is more resistant to enzymatic hydrolysis compared to the A-type structure (Buléon et al. 1998; Kwasniewska-Karolak et al. 2008). Although raw rice starch appears the typical A-type structure, recent studies indicate that the B-type structure could form during the digestion process through the rearrangement of amylose chains (Lopez-Rubio et al. 2008; Tawil et al. 2011). In order to determine whether such rearrangement is happen during the hydrolysis of 1 % raw rice starch by AmyP, the starch residue obtained after 4 h of hydrolysis was analyzed by X-ray diffraction. No B-type structure was observed (data not shown). Therefore, the resistance to amylolysis of the rice starch residue was likely caused by the morphology and the surface microstructure of the residue, rather than by the crystalline structure. It seemed logical to assume that the unique or susceptible sites on starch granule surface were rare in the rice starch residues.

In conclusion, this is the first report of the degradation ability on raw starch of an α-amylase from the marine bacterial subfamily GH13_37. The degradation ability of AmyP is very specific. It appears a preferential and rapid hydrolysis towards raw rice starch, which has not been described in any other known α-amylases.

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