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A starch-binding domain identified in α-amylase (AmyP) represents a new family of carbohydrate-binding modules that contribute to enzymatic hydrolysis of soluble starch

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ABSTRACT

A novel starch-binding domain (SBD) that represents a new carbohydrate-binding module family (CBM69) was identified in the α-amylase (AmyP) of the recently established alpha-amylase subfamily GH13_37. The SBD and its homologues come mostly from marine bacteria, and phylogenetic analysis indicates that they are closely related to the CBM20 and CBM48 families. The SBD exhibited a binding preference toward raw rice starch, but the truncated mutant ($AmyP_{\Delta SBD}$) still retained similar substrate preference. Kinetic analyses revealed that the SBD plays an important role in soluble starch hydrolysis because different catalytic efficiencies have been observed in AmyP and the $AmyP_{\Delta SBD}$.

1. Introduction

Glycoside hydrolases are generally modular enzymes, and very often contain non-catalytic ancillary domains referred to as carbohydrate-binding modules (CBMs), which provide catalytic modules with enhanced ability to bind onto specific polysaccharide surfaces. CBMs with affinity for insoluble raw starch are commonly referred to as starch binding domains (SBDs). SBDs having approximately 100 amino acid residues are present in about 10% of amylases and their related enzymes [1–3]. They are found at the C- or N-terminus of α-amylases, β-amylase, glucoamylase, and cyclodextrin glucanosyltransferase [3]. The SBD is known to bind raw starch, increasing the local concentration of substrate at the active site of the enzyme, and in some cases it has a disruptive function on the surface of the raw starch granule [4]. Therefore, raw starch-degrading enzymes generally possess SBDs [5]. At present sequence-based classification gives SBDs into the 10 following CBM families: CBM20, 21, 25, 26, 34, 41, 45, 48, 53 and 58 [2,6,7]. Noticeably, these SBDs mostly come from terrestrial organisms. Little is known about SBDs that are produced by marine organisms.

Recently, we isolated a novel α-amylase (EC 3.2.1.1) (AmyP) from a marine metagenomic library, which shows extremely low (no more than 20%) sequence similarity to other known amylases [8]. The discovery led to the identification of a new subfamily alpha-amylase family (GH13_37) that may be an independent clade of ancestral marine bacterial α-amylases. Currently, the subfamily GH13_37 contains 18 putative glycosidases that come mostly from marine bacteria (CAZy database, [6]). In addition to the AmyP, none of them has been biochemically characterized. AmyP was later shown to exhibit a unique and remarkable ability to preferentially and very rapidly digest raw rice starch, which has not been described in any other known α-amylases [9].

In this study, a C-terminal region of AmyP was identified as a novel SBD by adsorption characterization. The absence of alignment of the SBD sequence with that of any classified CBM indicates that this SBD defines a novel family of CBMs, which we propose to name CBM69. The SBD has an unexpectedly stronger effect on the catalytic activity toward soluble starch than that of raw starch, indicating that the SBD plays an important role in soluble starch hydrolysis, which broadens our understanding of the function of SBDs.

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2. Materials and methods

2.1. Sequence analysis

Conserved domain searches were performed using NCBI Conserved Domain Database [10]. The SBDs of the 10 CBM families were selected from the Carbohydrate-Active Enzyme (CAZy) database [see www.cazy.org] according to the previously reported classification [2,6,7,11–14]. A total of 18 sequences [2,7] represented the CBM20 and CBM48 families were extracted. All sequence alignments were performed using the CLUSTAL X [15] and then manually adjusted where required. The adjustment strategy was based on the approach described by Machovič et al. [16]. The evolutionary tree was calculated with the neighbour-joining method [17] implemented in the CLUSTAL X package using the final alignment. The tree was displayed with the program TreeView [18].

2.2. Construction of plasmids

Plasmid pET32a-*amyP* encoding the full length AmyP (GenBank accession number HM572234) was used as a PCR template [8]. DNA fragment corresponding to the 508 N-terminal amino acids of AmyP, named $AmyP_{\Delta SBD}$, was generated using the following primers: 5′-CCCCATATGTGCGATAGCGCTTTGA-3′ (*Nde*I site underlined) and 5′-CCCCTCGAGTGACGATGCAGCAGAAACC-3′ (*Xho* I site underlined). The SBD protein (131 amino acid residues) was obtained using primer 5′-CCCCATATGTTGATAGGTGAGGGTTTCA-3′ (*Nde*I site underlined) and primer 5′-CCCCTCGAGCGGAGACTTAGAGACCAT-3′ (*Xho* I site underlined). The resulting PCR products were digested with *Nde*I and *Xho*I and cloned into the pET28a vector (Novagen) with a C-terminal His-tag.

2.3. Gene expression and purification

Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells (Novagen). The transformed cells were grown in LB medium supplemented with kanamycin at 37 °C until the optical density at 600 nm of 0.5–0.7 was reached. At this point, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cells were incubated at 16 °C for 7 h to induce proteins. Cells were harvested and lysed by ultrasonication. The recombinant proteins were purified with one-step purification procedure using a HiTrap chelating HP column (GE Healthcare BioSciences).

2.4. Raw starch granules adsorption

The ability of proteins to adsorb to insoluble raw rice starch (or other raw starches as specified in the text) was determined as mentioned previously [19]. Briefly, raw rice starch was prewashed three times with Milli-Q water. 60 μl of AmyP (0.54 μmol), the $AmyP_{\Delta SBD}$ (1.4 μmol) or the SBD (3.9 μmol) was added to 0.6 ml of 50 mM sodium/potassium phosphate buffer (pH 7.5) containing 10, 20, 40, 60, 80, 100 and 120 mg of raw rice starch, respectively. The reactions were incubated at 4 °C for 1 h under gentle shaking, and then centrifuged at 12 000*g* for 5 min to pellet the starch and bound protein. Two 50 μl aliquots of each sample were removed and the amount of unbound protein was determined by using Bradford method with bovine serum albumin as a standard. Raw rice, corn, potato and wheat starch were purchased from Sigma. Raw mung and pea starch were purchased from Hengshui Fuqiao Starch Co., Ltd. (Hebei, China).

2.5. Enzyme activity assay

The activity of α-amylase was determined by measuring the amount of reducing sugar using 3′,5-dinitrosalicylic acid (DNS) as described by Miller [20]. Since the optimum pH and temperature of AmyP can be altered by changing the substrates [9], soluble starch (Sigma) and raw starch degrading activities were determined at 50 and 40 °C for 5 min, respectively. The reaction mixture contained 100 μl appropriately diluted purified enzyme and 500 μl 1% soluble starch (or other soluble polysaccharides) in 50 mM sodium citrate buffer (pH 6.5) or 500 μl 4% raw rice starch (or other raw starches) in 50 mM sodium/potassium phosphate buffer (pH 7.5). AmyP and the $AmyP_{\Delta SBD}$ were dosed at the same activity, that is, 0.1 U per mg of soluble substrate and 0.5 U per mg of insoluble raw starch. The activity unit was determined using soluble starch as substrate. All raw starches were washed three times with ice Milli-Q 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 [21]. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of reducing groups as glucose per min [22]. Amylose, amylopectin, pullulan and glycogen were purchased from Sigma.

2.6. Kinetics of reactions

Gelatinized rice starch was prepared as previously described [23]. Briefly, raw rice starch granules were suspended in 50 mM sodium citrate buffer (pH 6.5), and completely gelatinized by heating in a boiling water bath for 20 min. The flask was sealed to restrict water loss by evaporation during heating, and the flask and its contents were weighed both prior to and after 20 min in order to avoid any loss of volume. Suspension was freshly prepared and used immediately for each experiment. The Michaelis constant (K_m) of each enzyme was determined at 10 different starch concentrations (from 0 to 40 g/liter) at the optimal temperature and pH. Kinetic parameters were calculated by fitting initial velocities and substrate concentrations to the Michaelis–Menten equation by using the quasi-Newton minimization method (Microsoft Excel).

3. Results and discussion

3.1. Identification of the CBMxx family

Amino acid sequence alignments of AmyP and its four close homologues reveal that an extended region is located in the C-terminal region of AmyP, the putative glycosidase (CAG23328) of *Photobacterium profundum* (termed AMY_Phopr) and the putative glycosidase (CAV27328) of *Vibrio splendidus* (termed AMY_Vibsp), respectively (Fig. 1a). The three extended regions of about 130 amino acid residues show more than 62% sequence similarity to each other. The BLAST search results show the region of AmyP has the highest identity (72% over 108 amino acids) to the C-terminal region of the putative neopullulanase (WP_007465856) from marine bacterium *Photobacterium* sp. AK15, followed by 70–40% identities to the C-terminal or N-terminal region of many putative glycosidase from marine bacteria *Photobacterium* sp. strains and aquatic animal pathogens *Vibrio* sp. strains. All these *Vibrio* sp. strains are marine fish (or prawn, crab, rotifer) pathogens except *Vibrio rumoiensis* [24] and some unreported *Vibrio* sp. strains cannot identify whether they are marine pathogenic bacteria. None of these enzymes has been biochemically characterized. SBDs are generally found in raw starch-degrading enzymes and mostly placed in the C-terminal end of proteins. We guessed the C-terminal region of AmyP might be a SBD, although the domain displays no significant BLAST hit to SBD sequences in the CAZy database [6]. According to the CAZy modular assignment [6], HMMER [25] using Hidden markov models (HMMs) is another sequence comparison tool used to define enzyme family. There is still a lack of significant hits with HMMs derived from any known CBM sequences. Therefore, the

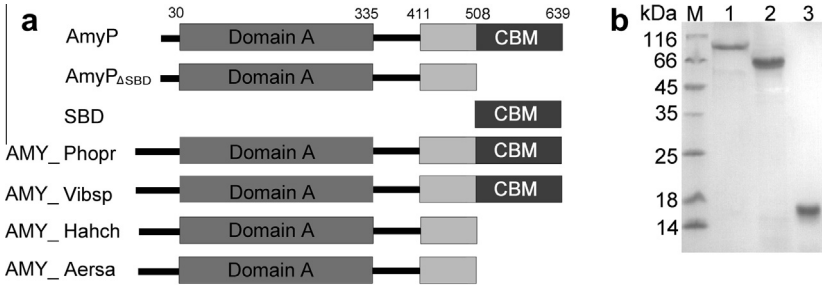

Fig. 1. (a) Schematic of AmyP and its four homologues: amino acid numbers corresponding to the module boundaries are shown above the schematic. The individual module constructs and their designations are also shown. The homologues from *P. profundum*, *V. splendidus*, *Hahella chejuensis* and *Aeromonas salmonicida* are abbreviated to AMY_Phopr, AMY_Vibsp, AMY_Hahch and AMY_Aersa, respectively. (b) SDS–PAGE gel of purified recombinant proteins: lane M, marker (kDa); lane 1, full-length AmyP; lane 2, truncated AmyP$_{\Delta SBD}$; lane 3, SBD.

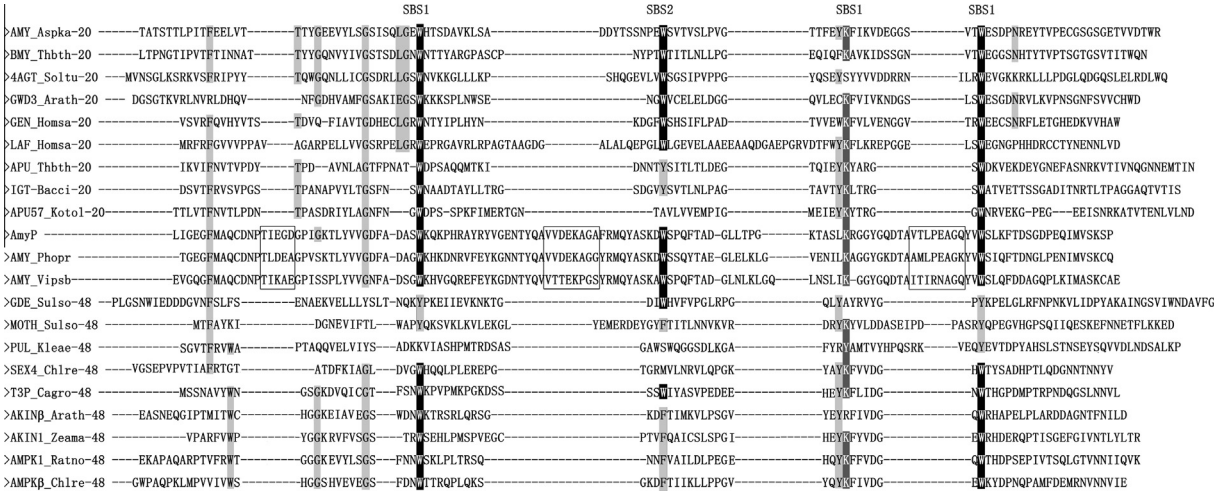

Fig. 2. Amino acid sequence alignment of the SBD of AmyP, its two homologues and the SBD representatives from CBM20 and CBM48 families. Three conserved tryptophanns involved in binding sites 1 and 2 are in black. The functionally important lysine of binding site 1 is in dark gray. The remaining conserved residues are in light gray, including the additional well-conserved phenylalanine in CBM20s and CBM48s. Boxed regions are the extra residues located at the SBD of AmyP. The abbreviations of the source proteins were referred to published data [2,7].

region of AmyP and its homologues will be designated as a new family of CBMs (family 69, CBM69).

3.2. The CBM69 family is evolutionarily related to the CBM20 and CBM48 families

Based on the previously reported classification of SBDs, the SBDs of CBM20, 21, 25, 26, 34, 41, 45, 48, 53 and 58 families were selected. Alignments of amino acid sequences between the SBDs from AmyP, AMY_Phopr and AMY_Vibsp and each of the 10 CBM families were performed individually (data not shown). Although the three SBDs are approximately 130 amino acids longer than the CBM20 and CBM48 representative members, some conserved residues of the CBM20 and CBM48 families, i.e. two starch-binding sites (SBS1 and SBS2) that are the best conserved in CBM20 and CBM48 [2], can be identified in the three SBDs (Fig. 2). The results show that the CBM69 family is closely related to the CBM20 and CBM48 families. The CBM20 family is the earliest-assigned and best characterized family. Recent studies have suggested that the relationship between CBM20 and CBM48 families is very close since there are representatives in both CBM families that possess an intermediate character [2,7,11]. However, the current knowledge of SBDs comes predominantly from terrestrial organisms. There are only several SBDs isolated from marine bacteria in the CAZy database, but none of them has been phylogenetically and biochemically characterized. Phylogenetic analysis of the CBM69 family is presented in Fig. 3, relative to related CBM20 and CBM48 families. The SBD of AmyP and its homologues form a clearly distinct group, and are positioned between CBM20 and CBM48 families. Previously, the border zone of the CBM20 and CBM48 families is occupied by the members lacking the SBS2 or SBS1 canonical binding site residues [2]. The location of the new CBM69 family possibly reflects the fact that these SBD are from marine bacteria rather than the terrestrial species. The results provide the new clues of the biodiversity and distribution of SBDs in the marine environment.

3.3. Construction and purification of truncated derivatives

To explore the function of the SBD, we used recombinant DNA techniques to generate the C-terminally truncated AmyP$_{\Delta SBD}$, and the SBD (Fig. 1a). During the expression in *E. coli* a large portion of the recombinant AmyP$_{\Delta SBD}$ and SBD remained insoluble despite various efforts to increase the yield of soluble proteins (data not shown). The soluble forms of AmyP$_{\Delta SBD}$ and SBD were purified to near homogeneity by Ni^{2+} affinity chromatography. The apparent molecular weights of AmyP$_{\Delta SBD}$ and SBD were slightly larger than the calculated molecular mass of 56 and 14 kDa, respectively (Fig. 1b), which were due to the His-tag® fused expression.

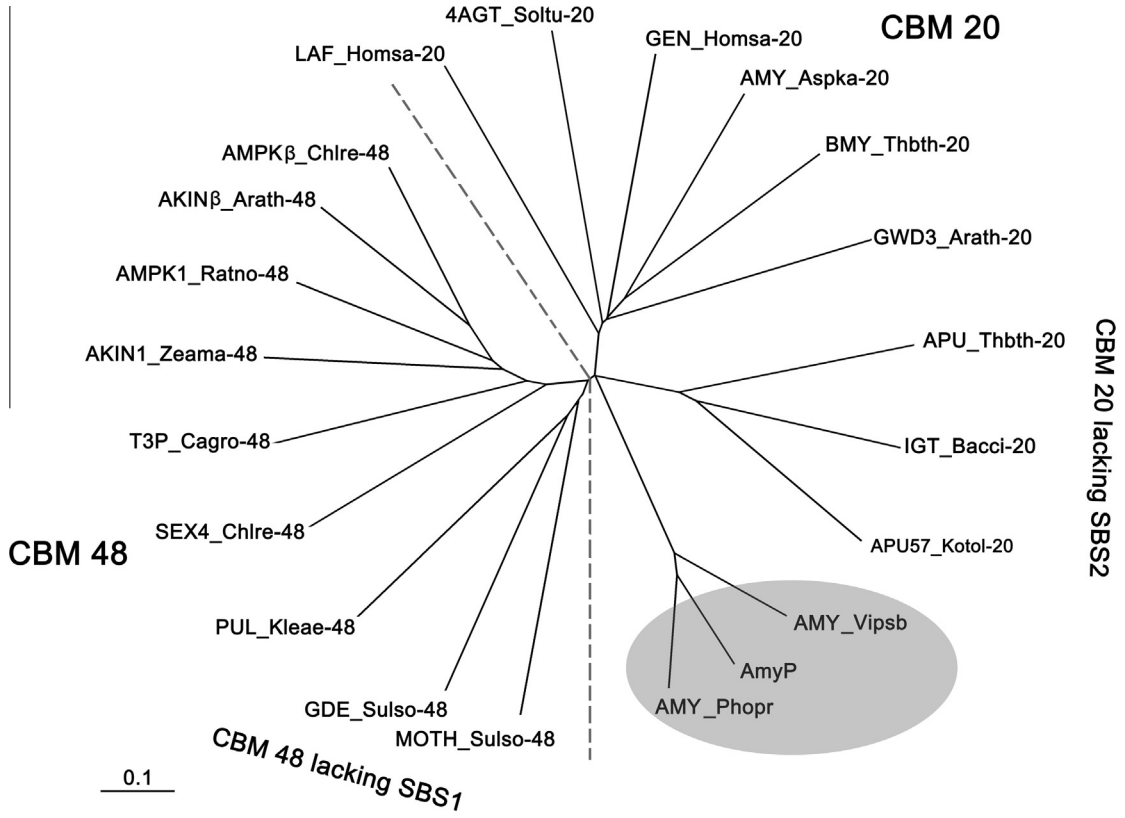

Fig. 3. Evolutionary tree of SBDs. The abbreviations of the source proteins are same in Fig. 2. A dashed line separates the CBM20 family from the CBM48. The SBDs from AmyP and its homologues are clearly distinguishable. The tree is based on the alignment made in CLUSTAL X of the complete SBD sequences (shown in Fig. 2), including the gaps. The scale bar indicates 0.1 amino acid replacements per site.

3.4. Ability to bind to raw starch

Adsorptions of AmyP, the AmyP$_{\Delta SBD}$ and the SBD to raw rice starch granules were assayed at various starch concentrations. The amount of bound protein as a fraction of the total protein versus mg of raw rice starch is plotted in Fig. 4. The SBD and AmyP displayed similar relative affinities for raw starch whereas the AmyP$_{\Delta SBD}$ was unable to bind to it. This suggested that the C-terminal region of AmyP was unambiguously defined as a SBD, and AmyP binding to substrate is very much dependent upon the SBD. The fact that the SBD alone had the highest affinity for raw rice starch may be due to more facile interactions between such a small protein and a large starch granule compared with the full-length AmyP. Similar adsorption behaviors were also seen in an α-amylase (SusG), truncated SusG and its SBD from *Bacteroides thetaiotaomicron* [19].

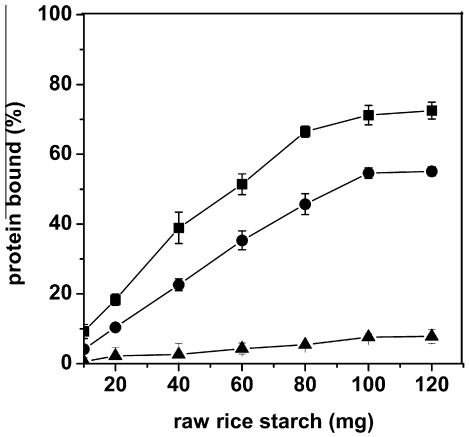

Fig. 4. Protein binding to insoluble raw rice starch. Fraction of protein bound versus mg of starch was plotted. The binding of AmyP (●), the AmyP$_{\Delta SBD}$ (▲), and the SBD (■) to various concentrations of starch was performed as described in Section 2.

AmyP is efficient in hydrolyzing various raw starches from terrestrial plants, moreover, it rarely exhibited a preferential hydrolysis toward raw rice starch [9]. Therefore, a maximal fraction of bound protein was used to evaluate the ability of the SBD to interact with various raw starch granules. At the tested protein and starch concentrations, the maximal fraction of bound protein toward raw rice starch, raw corn starch and raw mung starch was 72 ± 2.4% (Fig. 4), 31 ± 1.8% and 10 ± 3.3%, respectively. The SBD is weak in the binding of raw wheat starch, raw potato starch and raw pea starch, and a precise maximal fraction of bound protein could not be determined by using the method of quantifying protein. However, the abilities of the SBD to bind to raw wheat starch, raw potato starch and raw pea starch were observed by using the more sensitive affinity gel electrophoresis (data not shown). The difference in the binding affinities of the SBD was essentially consistent with the unusual ability of AmyP to attack various raw starches, implying that the SBD module and its attached enzyme have a closely coevolutionary relationship.

3.5. Effect of the SBD on enzymatic hydrolysis

To further investigate the functional interdependency of the SBD and the full length AmyP, the substrate specificity and hydrolysis curve of the AmyP$_{\Delta SBD}$ were measured and compared with those of AmyP. By dosing the two proteins at the same activity per mg polysaccharide, their abilities of hydrolysis on polysaccharide can be strictly compared. Although the AmyP$_{\Delta SBD}$ was unable

to bind to raw starches, it was significantly active on all polysaccharides prepared according to the substrate range of AmyP, and only showed a varying degree of decrease in specific activity compared to AmyP (Table 1). With respect to the raw starch substrates, the AmyP$_{\Delta SBD}$ still exhibited the unique ability to preferentially digest raw rice starch. The results indicated that the SBD does not play a functional role in determining substrate specificity.

Another unusual feature of AmyP is a very rapid digestion of raw rice starch, requiring only 4 h to complete the hydrolysis [9]. At the tested enzymatic concentration (0.5 U per mg of raw starch), the hydrolysis curve of the AmyP$_{\Delta SBD}$ on 4% raw rice starch was compared to that of AmyP (Fig. 5). The reducing sugars released by the AmyP$_{\Delta SBD}$ were less than about one half amount that determined for AmyP. The main hydrolysis of the AmyP$_{\Delta SBD}$ occurred during the early 1 h, and reducing sugars were not obviously increased after reaction for 3 h, suggesting that the SBD is not involved in the speed of digestion.

Although the SBD makes no contribution to the substrate specificity and the speed of digestion, it is interesting to note that AmyP exhibited a 4.4-fold higher specific activity toward the soluble starch than the truncated AmyP$_{\Delta SBD}$, whereas substitution of soluble starch with raw rice starch decreased the ratio from 4.4 to 2.1 (Table 1). The data strongly imply that the presence of SBD in AmyP has a stronger effect on the catalytic activity toward soluble starch than that of raw starch. To eliminate the influence of starch source on the activity and confirm further the role of the SBD with regards to starch hydrolysis, the kinetic parameters of both enzymes on gelatinized and raw rice starch were determined (Table 2). As expected, the catalytic efficiency (k_{cat}/K_m) of AmyP was 4.3-fold higher than that of the AmyP$_{\Delta SBD}$ with gelatinized rice starch as the substrate, while with respect to raw rice starch, the catalytic efficiency (k_{cat}/K_m) of AmyP was decreased to 2.3-fold higher than that of the AmyP$_{\Delta SBD}$. The increase in catalytic efficiency resulted dominantly from an increase in k_{cat}, since the AmyP$_{\Delta SBD}$ had only slightly higher K_m values for both gelatinized and raw rice starch than AmyP. The data agreed well with the results obtained in the substrate specificity assays. This disparity between soluble and raw starch digestion was surprising, which indicates that the function of the SBD in AmyP is not simply to concentrate the enzyme on the substrate, a role typically assigned to SBDs. The SBD may play an important role in soluble starch hydrolysis. In addition, it is important to note that AmyP is from a marine metagenomic library constructed from subsurface sediments [8]. The exclusive function of the SBD probably reflects a fact that soluble starch is more like an unknown nature substrate of AmyP, after all, no starch plant grows in dark and cold deep sea.

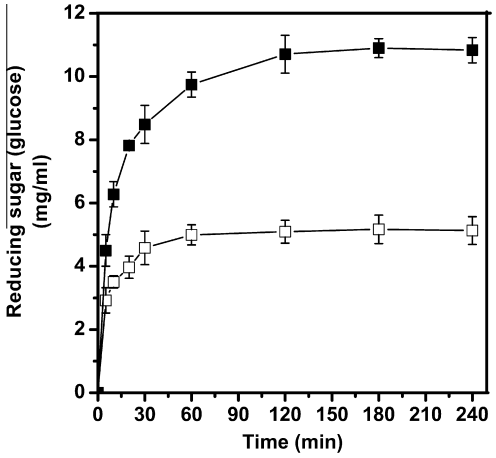

Fig. 5. Digestion of 4% raw rice starch by AmyP (■) and the AmyP$_{\Delta SBD}$ (□) as measured by release of reducing sugars. Incubations were carried out at pH 6.5 and 40 °C.

Table 2
Kinetic parameters of AmyP and the AmyP$_{\Delta SBD}$ using gelatinized and raw rice starch as the substrates.

	AmyP	AmyP$_{\Delta SBD}$	Ratio (AmyP:AmyP$_{\Delta SBD}$)
Gelatinized rice starch			
K_m (mg/ml)	2.5	2.9	0.86
k_{cat} (/s)	1.53	0.41	3.7
k_{cat}/K_m (s/mg/ml)	0.61	0.14	4.3
Raw rice starch			
K_m (mg/ml)	13.4	15.9	0.84
k_{cat} (/s)	0.57	0.30	1.9
k_{cat}/K_m (s/mg/ml)	0.043	0.019	2.3

It is well known that the function of SBD is associated with insoluble forms of starch. Deletion of SBD usually results in a dramatic decrease in raw starch hydrolysis [26,27], even causing no activity toward raw starch [28]. Hydrolytic activity of the truncated enzyme on soluble starch consequently decreases, but the decreasing degree of activity is much less than that of raw starch [26], which can be explained as the results of a slight damage of complete protein structure, not necessarily caused by a role of SBD in soluble starch hydrolysis. In several amylases, SBD even seems to actually hinder degradation of soluble starch, because the truncated enzyme displays an obvious increased level of

Table 1
Specific activities of AmyP and the AmyP$_{\Delta SBD}$ toward various substrates.

Test substrate	AmyP		AmyP$_{\Delta SBD}$		Ratio of activity (AmyP: AmyP$_{\Delta SBD}$)
	Activity (U/mg)	Relative activity (%)	Activity (U/mg)	Relative activity (%)	
Soluble substrate[a]					
Soluble starch	453.5 ± 10.7	100	103.2 ± 4.8	100	4.4
Amylose	140.6 ± 7.2	31	59.8 ± 1.6	58	2.3
Amylopectin	68.1 ± 5.5	15	32.3 ± 2.1	31	2.1
Pullulan	45.3 ± 3.8	10	17.5 ± 2.9	17	2.6
Glycogen	13.1 ± 2.3	3	7.4 ± 1.3	7	1.8
Insoluble raw starches[b]					
Rice	119.2 ± 1.6	100	55.5 ± 1.4	100	2.1
Corn	39.6 ± 2.1	33	20.7 ± 1.5	37	1.9
Wheat	7.0 ± 0.9	6	3.8 ± 0.8	7	1.8
Potato	12.3 ± 1.1	10	4.5 ± 1.3	8	2.7
Mung	16.8 ± 2.0	14	5.4 ± 0.4	10	1.7
Pea	8.6 ± 1.3	7	3.7 ± 0.6	7	2.3

[a] Substrate concentration, 1%.
[b] Substrate concentration, 4%.

Table 3
Effects of the SBD of AmyP and other SBDs on hydrolytic properties.

Enzyme	CBM family		Substrate		Annotation of activity data	The effect of SBD on hydrolysis	Origins	Reference
			Soluble starch	Raw starch				
α-Amylase (AmyP)	CBM20	WT $AmyP_{\triangle SBD}$	453.5 ± 10.7 103.2 ± 4.8	119.2 ± 1.6 55.5 ± 1.4	Activity (U/mg enzyme)	A stronger effect on soluble starch hydrolysis than raw starch hydrolysis	Unknown marine bacterium	Present work
α-Amylase	CBM26	WT △SBD	100 20	100 0	Relative activity (%)	Prerequisite for raw starch hydrolysis	*Lactobacillus amylovorus*	[28]
Glucoamylase	CBM20	A fusion protein of barley α-amylase and SBD Barley α-amylase	0.27 0.14	0.7 0.07	Activity (U/nmol enzyme)	A weaker effect on soluble starch hydrolysis	*Aspergillus niger*	[26]
α-Amylase (SusG)	CBM58	WT △SBD	100 ± 12.7 162 ± 5.9	100 ± 5.7 29 ± 1.4	Relative activity (%)	Hinder degradation of soluble starch	*Bacteroides thetaiotaomicron*	[19]
α-Glucan, water dikinase	CBM45	WT △SBD	5.8 ± 0.2 12.7 ± 0.7	ND ND	Activity (milliunits/mg enzyme)	Hinder degradation of soluble starch	Potato (*Solanum tuberosum*)	[29]
α-Amylase	CBM25	WT △SBD	2.9 3.8	13.5 1.5	Soluble starch: k_{cat}/k_m ($s^{-1}mg^{-1}l$); raw starch: digestion rate (%)	Hinder degradation of soluble starch	*Bacillus* sp. 195	[27]
α-Amylase	CBM20	WT △SBD	200.3 207.1	11 11	Soluble starch: activity (U/mg enzyme); raw starch: 10-h digestion rate (%)	No effect	*Bacillus* sp. TS-23	[30]
Amylopullulanase	CBM20	WT △SBD	1.194×10^{10} 1.174×10^{10}	1.186×10^{9} 1.175×10^{9}	Activity (U/mole enzyme)	No effect	*Thermoanaerobacter ethanolicus* 39E	[31]

activity on soluble starch compared to wild-type enzyme [19,27,29]. Thus far, none of the biochemical analyses has identified an essential role for SBD on soluble forms of starch. Various effects of SBDs on hydrolysis of soluble starch and raw starch were summarized in Table 3. It can be seen that only the SBD of AmyP makes more significant contribution to soluble starch hydrolysis rather than to raw starch hydrolysis.

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