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Surface binding sites in amylase have distinct roles in recognition of starch structure motifs and degradation

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ABSTRACT

Carbohydrate converting enzymes often possess extra substrate binding regions that enhance their activity. These can be found either on separate domains termed carbohydrate binding modules or as so-called surface binding sites (SBSs) situated on the catalytic domain. SBSs are common in starch degrading enzymes and critically important for their function. The affinity towards a variety of starch granules as well as soluble poly- and oligosaccharides of barley α-amylase 1 (AMY1) wild-type and mutants of two SBSs (SBS1 and SBS2) was investigated using Langmuir binding analysis, confocal laser scanning microscopy, affinity gel electrophoresis and surface plasmon resonance to unravel functional roles of the SBSs. SBS1 was critical for binding to different starch types as K_d increased by 7–62-fold or was not measurable upon mutation. By contrast SBS2 was particularly important for binding to soluble polysaccharides and oligosaccharides with α-1,6 linkages, suggesting that branch points are key structural elements in recognition by SBS2. Mutation at both SBS1 and SBS2 eliminated binding to all starch granule types tested. Taken together, the findings indicate that the two SBSs act in concert to localize AMY1 to the starch granule surface and that SBS2 works synergistically with the active site in the degradation of amylopectin.

1. Introduction

Barley α-amylase isozymes 1 and 2 (AMY1 and AMY2, EC 3.2.1.1) together with β-amylase, α-glucosidase and limit dextrinase are involved in mobilization of endosperm starch in germinating barley seeds [1]. AMY1 and AMY2 hydrolyse 1,4-α-D-glucosidic linkages in starch and related poly- and oligosaccharides and share about 80% sequence identity [2,3]. The targeting and binding of amylolytic enzymes to starch granules commonly depend on non-catalytic binding sites on auxiliary domains known as carbohydrate binding modules (CBMs), in this case starch binding domains (SBDs), or on surface binding sites (SBSs) situated on the catalytic domain – all facilitating efficient catalysis [4–6]. Approximately 7% of all glycoside hydrolases in the CAZy database [7] (www.cazy.org) contain at least one CBM [8], removal of which can reduce, or in some cases abolish, binding and activity towards insoluble substrates [6]. Thus deletion of the SBD (CBM20) from *Aspergillus niger* glucoamylase dropped activity 100-fold on starch granules [9], while fusion of this SBD to AMY1 resulted in six-fold increase in affinity and higher activity for starch granules [10].

SBSs are much more challenging to identify than CBMs as they typically are discrete sites belonging to the catalytic domain rather than an independent module. The vast majority of SBSs discovered to date were identified in crystal structures of enzymes in complex with substrates or substrate-analogues. SBSs, similarly to CBMs, can target the enzyme to its substrate, but are also found to have other functions, e.g. feeding a sugar chain into the active site and serving in allosteric regulation or in substrate disruption [5]. Surprisingly, SBSs and CBMs probably have complementary roles since about 25% of the enzymes known to have an SBS also contain a CBM [4]. About half of these SBS-containing enzymes belong to glycoside hydrolase family 13 (GH13; CAZy designation [7]) also called the α-amylase family.

Crystal structures of AMY1 and AMY2 with bound maltoheptaose, acarbose (a pseudotetrasaccharide inhibitor) or methyl $4^{I},4^{II},4^{III}$-trithiomaltotetraoside revealed two SBSs, originally named "the starch granule-binding site" [11] and "the pair of sugar tongs" [12–14], and now referred to as SBS1 and SBS2, respectively

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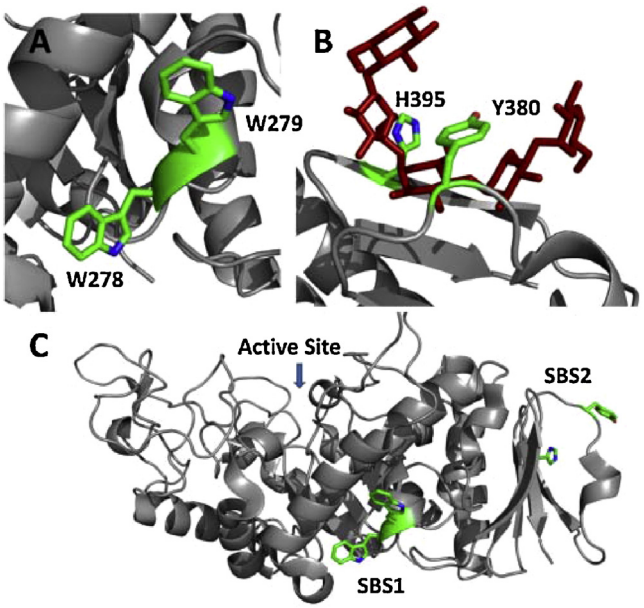

Fig. 1. The SBSs of AMY1. (A) SBS1 (PDB: 1HT6) with W278 and W279, (B) SBS2 Y380 and H395 in complex with maltoheptaose (PDB: 1RP8), (C) global structure of AMY1 (PDB:1HT6) with the SBS residues (highlighted in green) and the active site indicated. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

(Fig. 1). SBS1 has two tryptophans (W278; W279) exposed on the surface of the catalytic $(\beta/\alpha)_8$ barrel, whereas SBS2 from the C-terminal β-sheet domain includes a tyrosine (Y380) and a histidine (H395), both of which are required for binding at this site [15]. Mutational analysis showed both SBSs are critical for adsorption onto, and hydrolysis of barley starch granules and that SBS1 has four fold higher affinity than SBS2 [16,17]. AMY1 dual site SBS mutants (W278A/Y380A; W279A/Y380A; W278A/W279A/Y380A) lost ability to bind to starch granules and their catalytic efficiency decreased by 60–400-fold compared to wild-type [17]. SBS1 and SBS2 moreover participate in the multiple attack mechanism and modulate substrate binding energies along the active site cleft as analysed by subsite mapping [17]. Recently SBS2 was found to have a key role in hydrolysis of amylopectin [18].

Normal starch granules consist of approximately 25% α-1,4 linked amylose (AM) that is essentially linear and 75% amylopectin (AP) having an α-1,4 backbone carrying α-1,6 branch points. AM and AP are deposited as alternating amorphous and crystalline layers. The A-type crystalline polymorph, typical of cereal endosperm starches (here represented by barley and maize starches) forms densely packed double helical segments, while the B-type found in tuber and leaf starches (here represented by potato starch) has a looser packing density and a substantial amount of structured water. The C-type crystalline polymorph, commonly found in legume seeds (here represented by pea starch), is a mixture of A- and B-types. The organization of AM in the granule is less clear [19] and its impact on AP packing differs at low to moderate versus high AM levels [20,21], high AM varieties possessing a V_h-type single helical polymorph often complexed with lipids [22,23].

The surface of starch granules is the initial substrate for amylases. Atomic force microscopy (AFM) studies [24] indicate that so-called blocklet structures, which vary immensely among different starch types, are the dominant organizational feature at the granule surface. Blocklets are crystalline regions of AP, punctuated by hair like extensions of either AM or AP and are likely important points of enzyme recognition. High AM and B-type starches are generally more resistant to amylolytic attack than the A-type [25,26]. Hence, AP-only, i.e. waxy (wx) and normal maize starch granules were both readily degraded by a fungal α-amylase, while high AM maize starch was more resistant [27]. Recently, an AM-only barley starch showed combined glucoamylase and α-amylase resistance [28]. For maize starch, evidence was furthermore provided that hydrolytic sensitivity depends on long-range structural effects controlling enzyme diffusion to granule surfaces [29]. Finally tuber starches are slightly phosphorylated, forming weak points prone to degradation due to local hydration and amorphisation [30].

In order to explore the roles of SBSs in the breakdown of starch granules it is necessary to examine the interplay between the SBS and the active site. To do this, analysis has been performed on the ability of AMY1 wild-type and SBS mutants to interact with different starches and starch components. The results demonstrate SBS1 is the key anchor to the starch granule while SBS2 acts in concert with the active site, achieving efficient hydrolysis of substrate chains.

2. Materials and methods

2.1. Materials

Normal potato, maize, rice, and pea; wx (high AP) and high AM maize starches were from KMC (Brande, Denmark). Low phosphate potato starch was from a transgenic glucan, water dikinase antisense suppressor line [31]. High AM/high phosphate and high AP potato starch were from transgenic starch branching enzyme suppressor lines [32] and a granule bound starch synthase suppressor line (Lyckeby Stärkelse, Sweden), respectively. Barley starch granules were from Primalco (Finland) and chemicals from Sigma–Aldrich (St. Louis, MO) unless otherwise stated.

2.2. Protein production and purification

AMY1 wild-type [33] and mutants D180A [13], Y380A [16], W278A/W279A, W278A/Y380A, and W279A/W380A were produced as described [17]. Enzymes with intact SBS2 were purified on β-CD-Sepharose as described [34] and SBS2 mutants by anion exchange chromatography (ResourceQ 6 mL; GE Healthcare) [16]. Fractions with pure α-amylase (as judged by SDS-PAGE; NuPage 4–12% Bis-Tris, Invitrogen, Carlsbad, CA) were combined, concentrated to ~1 mg/mL (Centriprep, 10 kDa cut-off, Millipore), dialyzed against 10 mM MES pH 6.8, 25 mM $CaCl_2$, added NaN_3 to 0.02% (w/v) and stored (4 °C).

2.3. Starch granule binding assays

Enzyme (5–10 nM) and starch granules were mixed (4 °C) in 20 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, 0.005% (w/v) BSA, final volume 500 μL, in 1.5 mL Eppendorf tubes, for 30 min with constant rotation and centrifuged (20,800 × *g*, 4 °C, 10 min). Supernatants were transferred to new Eppendorf tubes and kept on ice. Fourteen starch granule concentrations (strong, medium, weak affinity regimes: 0.1–20; 0.1–40; 0.5–100 mg/mL) were used (in triplicate). Unbound enzyme was quantified (using iBS activity assay, see Section 2.4) and data were fitted to Langmuir adsorption isotherm (Eq. (1)) [16,17]

$$B = \frac{B_{max}[S]}{K_d + [S]} \quad (1)$$

where *B* is the fraction of bound enzyme, [*S*] the concentration of starch granules, B_{max} the maximum bound fraction, and K_d the

dissociation constant. For waxy corn and rice starch granules data were fitted to a modified Langmuir adsorption isotherm (Eq. (2))

$$B = \frac{B_{max}[S]}{K_d + [S] + ([S]/K_S)} \quad (2)$$

where B, $[S]$, and K_d are as in Eq. (1) and K_s is the substrate inhibition constant.

2.4. Insoluble blue starch (iBS) assay

Enzyme (300 μL) reacted with 6.25 mg/mL iBS (customer preparation, Pharmacia) in 20 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, 0.005% (w/v) BSA (final 800 μL), incubated (37 °C; 15 min), stopped by 0.5 M NaOH (200 μL) and centrifuged (20800 × *g*, 4 min). Supernatants (300 μL) were transferred to a 96-well microtiter plate and absorbance measured at 620 nm (PowerWave XS, BIO-TEK Instruments GmbH, Germany) [16].

2.5. Fluorophore labeling of AMY1 and mutants

Enzyme (1.0 mg/mL) in 12 mM β-CD, 0.1 M sodium bicarbonate, pH 8.3, 25 mM $CaCl_2$, 0.02% NaN_3 was labelled (RT; 60 min) by 10-fold molar excess Fluorescein-5-EX succinimidyl ester (Invitrogen) and separated from remaining reagent (Zeba™ Desalt Spin Columns 0.5 mL; Thermo Fisher Scientific, Rockford, IL) in 20 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, 0.02% NaN_3. The average degree of labelling was about 0.5 Fluorescein-5-EX group/enzyme molecule as calculated from absorbance at 280 and 494 nm (Invitrogen instructions). The specific activity (iBS) was unchanged by the labelling.

2.6. Confocal laser scanning microscopy (CLSM)

Starch granules washed twice by centrifugation (13,000 × *g*, 10 min) in 20 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, and 0.005% BSA (5.7 mg/mL) were mixed with fluorescein-5-EX-AMY1 (final 260 nM) on the slide immediately before CLSM (TCS SP2, Leica Microsystems, Wetzlar, Germany) at room temperature. The instrument was equipped with an argon laser, the objective HC PL APO CS 20.0 × 0.70 IMM/COR, and a spectral filtering system allowing free selection of wavelength interval. A 488 nm laser line was used for excitation, beam splitter: TD 488/543/633, and light was detected between 500 and 550 nm. A laser power of 25% was maintained during acquisition of all images. The gain was varied to prevent saturation of the detector and to ensure comparable fluorescence intensities in all images. During image acquisition each line was scanned 16 times and averaged to reduce noise.

2.7. Affinity gel electrophoresis (AGE)

PAGE (12% separating, 4% stacking polyacrylamide gel; GE Healthcare) was performed in running buffer (50 mM Tris-Borate pH 8.7) in gels cast without (control) or with 0.1% glycogen (dissolved in water), 0.1% AP, 0.05% AM or 0.05% pullulan (dissolved in DMSO, diluted with hot water to final concentration at 2–7% DMSO). DMSO did not affect enzyme polysaccharide interactions (not shown) or polymerization with ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED). Proteins (0.5 mg/mL) were mixed with 5× loading buffer (running buffer with glycerol 5% and 1 mg/mL bromphenol blue); NativeMark™ protein ladder (Invitrogen) was the reference. Gels were run (55 V; 20 h; 4 °C) and CBB stained. The relative mobility was calculated from the ratio of protein migration distance in polysaccharide and control gels, divided by the corresponding ratio for a reference protein in the ladder with the most similar migration in the control. Such reference-corrected values of relative mobility removed any non-specific effects and were used to compare retardation of wild-type and mutants.

2.8. Surface plasmon resonance (SPR)

AMY1 wild-type and SBSs mutants were biotinylated and immobilized on streptavidin (SA) chips (Biacore® T100; GE Healthcare) to approximately 2500 response units (RU) [17]. Binding of β-CD, maltoheptaose, or α-limit dextrin DP10 (in-house prepared as by Roberts and Whelan [35]) was measured by passing 25 μM to 10 mM limit dextrin/maltoheptaose or 2.5 μM to 3 mM β-CD over bound proteins at 25 °C. Signals from a reference cell and a buffer blank in the sample cell were subtracted from sample signals. K_d and maximum binding response (B_{max}) for interactions were obtained by a two-site binding model for AMY1 wild-type or D180A interacting with β-CD as analyte and a one site binding model in all other cases (BiaEvaluation software; GE Healthcare). The binding stoichiometry was calculated by dividing the ratio of the maximum binding response to the bound protein response by the ratio of the analyte mass to the protein mass.

3. Results

3.1. Starch granule binding assays

AMY1 wild-type bound strongly (K_d = 0.12–0.76 mg/mL) to starch granules of four botanical origins, i.e. rice, followed by maize, barley and pea, including mutant maize of altered granule structure, while the affinity for normal and transgenic potato starch granules was much lower (K_d = 2.2–16 mg/mL) (Table 1). Generally the maize starch granules provided high affinity sites for AMY1. Within the this system, AMY1 had lower affinity for high AM maize compared to wx and normal maize starch granules demonstrating the importance of amylopectin for high affinity.

Reduced affinity of SBS mutants for the different starch variants gave insight into determinants for binding at SBS1 and SBS2. The relative affinity loss towards maize and barley starches by SBS2 mutation was comparable, while the SBS1 mutant showed two-fold greater loss for normal and wx maize starches than for barley starch. By contrast, the SBS1 mutant elicited lower relative affinity loss for high AM maize compared to barley starch, suggesting the motif recognized by SBS1 is more prominent in normal and wx maize starch granules, i.e. possibly A-type polymorphs, than in the high AM B/V_h crystalline variant.

Interestingly, potato starches displayed a larger variation in K_d than maize starches. Interaction of AMY1 with low phosphate and high AM potato starches had 7- and 3-fold higher K_d, respectively, than with normal or high AP potato starch. This confirmed the importance of organised/crystalline AP for binding, as low phosphate starch has decreased AP content [21,31] and phosphate is an important modulator of AP crystallinity [30]. While both SBS1 and SBS2 mutations led to less loss in affinity with normal potato compared to barley starch, the binding capacity was significantly reduced for both mutants and the wild-type affinity was already considerably lower for potato than for barley starch. Binding to high AP and high AM potato starch was essentially destroyed for the SBS1 mutant and K_d could not be determined. For the SBS2 mutant, affinity for high AM potato starch was reduced by the same factor as for normal starch, while affinity towards the high AP starch was reduced by about two-fold more, in line with the relative affinity reduction for this mutant on barley starch.

Table 1
Affinity of AMY1 wild-type and mutants for starch granules.

AMY1	Starch granules		K_d (±SD) mg/mL	B_{max} (±SD)	K_s (±SD) mg/mL	Ratio to wild-type K_d
Wild-type						
	Barley	- Normal[a]	0.64 ± 0.06	0.98 ± 0.02	–	–
	Maize	- Normal	0.24 ± 0.07	0.99 ± 0.03	–	–
		- High AM	0.46 ± 0.06	1.04 ± 0.02	–	–
		- Wx	0.18 ± 0.04	1.01 ± 0.03	166 ± 29	–
	Potato	- Normal	2.3 ± 0.4	0.96 ± 0.04	–	–
		- High AP	2.2 ± 0.1	0.99 ± 0.01	–	–
		- Low phosphate	16 ± 2	1.04 ± 0.01	–	–
		- High AM	7.3 ± 0.4	1.06 ± 0.01	–	–
	Rice	- Normal	0.12 ± 0.01	0.97 ± 0.01	46 ± 4	–
	Pea	- Normal	0.76 ± 0.04	1.00 ± 0.01	–	–
Y380A (SBS2)						
	Barley	- Normal[a]	5.9 ± 0.5	0.90 ± 0.05	–	9.2
	Maize	- Normal	2.51 ± 0.09	0.85 ± 0.01	–	10.4
		- High AM	4.7 ± 0.4	1.01 ± 0.01	–	10.2
		- Wx	1.5 ± 0.3	0.86 ± 0.07	103 ± 53	8.3
	Potato	- Normal	11.1 ± 0.8	0.68 ± 0.02	–	4.8
		- High AP	18 ± 3	0.65 ± 0.04	–	8.2
		- Low phosphate	n.d.	n.d.	–	–
		- High AM	32 ± 6	1.00 ± 0.07	–	4.4
W278A/W279A (SBS1)						
	Barley	- Normal[b]	22 ± 2	0.76 ± 0.01	–	34.4
	Maize	- Normal	15 ± 2	0.78 ± 0.01	–	62.5
		- High AM	9.4 ± 0.3	1.02 ± 0.01	–	20.4
		- Wx	11 ± 4	1.1 ± 0.2	30 ± 6	61.1
	Potato	- Normal	17 ± 8	0.36 ± 0.06	–	7.4
		- High AP	no binding	–	–	–
		- Low phosphate	n.d.	n.d.	–	–
		- High AM	>100	1.0 ± 0.2		–

n.d., not determined.
[a] [16].
[b] [17].

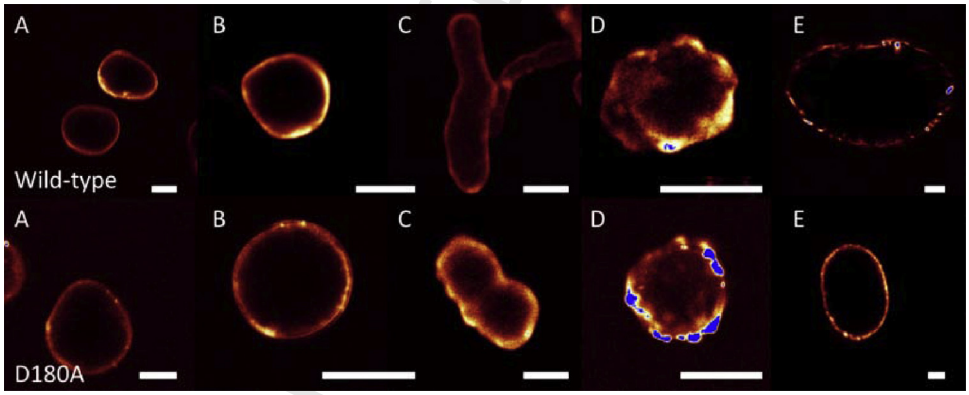

Fig. 2. Adsorption of AMY1 wild-type (top) and the catalytically inactive mutant D180A (bottom) to a selection of different starch granules. (A) Barley, (B) normal maize, (C) normal potato, (D) high AM maize, (E) low AM (wx) maize. See methods (Sections 2.5 and 2.6) for further details. The white calibration bars represent 10 μm.

3.2. Confocal laser scanning microscopy (CLSM)

Binding of fluorophore-labelled AMY1 on starch granule surfaces was visualized by images recorded within 10 min after mixing. Adsorption to the granules appeared instantaneous, yielding an evenly distributed fluorescence on the surface. Minor starch hydrolysis was observed due to the high enzyme concentration required for detection, which limited the time for collection of images. AMY1 wild-type and the inactive mutant D180A AMY1 showed identical binding distributions; hence starch hydrolysis had no detectable impact on the binding pattern (Fig. 2). Progressively longer incubation revealed different stages in starch granule degradation (Fig. 3). Eventually the enzyme infiltrated the starch granule and was observed in concentric layers, in accord with previous data on starch granule hydrolysis by amylase [36] showing amorphous layers were degraded in the early stages and access to these amorphous layers being favoured by the formation of cavities. The fluorescence was most intense in cavities indicating the affinity of AMY1 is higher towards these regions than the surface. CLSM showed single SBS mutants and wild-type AMY1 to be evenly distributed on the surface of barley starch granules (Fig. 4).

Remarkably, the dual SBS1 and SBS2 mutants W278A/Y380A and W279A/Y380A only bound to damaged areas of the granules and not on intact surfaces (Fig. 5). CLSM for these mutants, in contrast to the other images reported here, were recorded using maximum laser intensity to confirm that they indeed did not accumulate on the granule surfaces. These results demonstrate that the active site only recognizes structures in the interior of the granules and thus adsorption to the granule surface is exclusively guided by SBS1 and SBS2. Besides mutagenesis, β-CD can modify

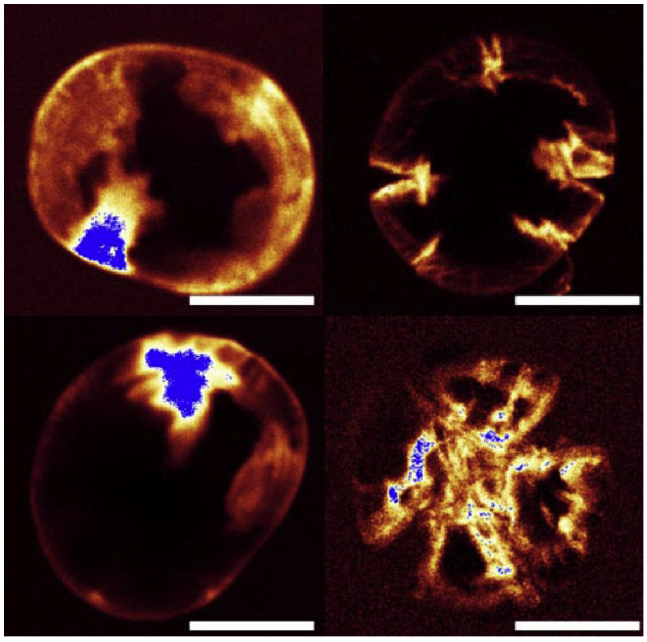

Fig. 3. Barley starch granules after 15–20 min incubation with active AMY1 wild-type. By this time point the fluorescence intensity inside the granule increased drastically compared to the surface binding indicating that the interior had a higher density of "binding sites" accommodating AMY1. The same phenomenon was also observed immediately after mixing the fluorophore-labelled enzyme with damaged starch granules or granules containing scratches. See methods (Sections 2.5 and 2.6) for further details. The white calibration bars represent 10 μm.

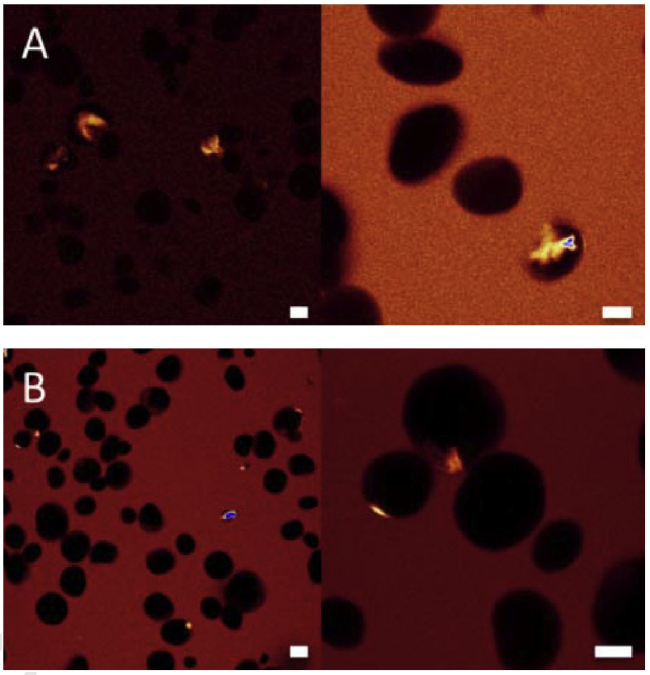

Fig. 5. Visualization of AMY1 dual surface binding site mutants interacting with barley starch granules. (A) W278A/Y380A (SBS1 and SBS2), (B) W279A/Y380A (SBS1 and SBS2). Binding was only observed on damaged surface areas of the starch granules. See methods (Sections 2.5 and 2.6) for further details. The white calibration bars represent 10 μm.

SBS function (Fig. 4) as it binds to both SBSs [16,37], but not the active site. SBS1 has 20-fold lower affinity than SBS2 for β-CD (K_d of 1.4 and 0.07 mM, respectively) [17]. AMY1 in the presence of 0.5 mM β-CD, selectively blocking SBS2, showed diminished binding, with some reduction in SBS1 mutant binding and no effect for the SBS2 mutant as expected. Blocking of both surface sites with 10 mM β-CD resulted in binding only at damaged regions of the starch granule, thus demonstrating active site interaction with damaged granule surfaces for both wild-type and mutant enzymes.

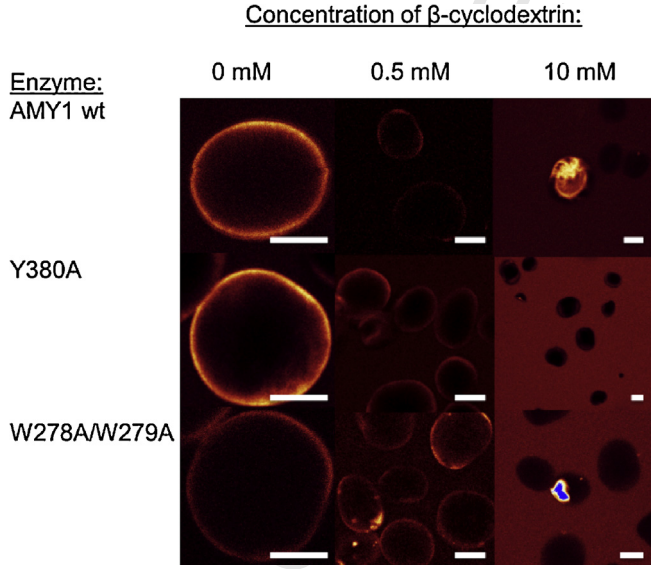

Fig. 4. Interaction of AMY1 wild-type and SBS mutants with barley starch granules in the presence and absence of β-cyclodextrin. Enzymes were incubated the starch granules and imaged immediately by CLSM. See methods (Sections 2.5 and 2.6) for further details. The white calibration bars represent 10 μm.

AMY1 was evenly distributed (Fig. 2) on high AM maize starch in contrast to normal and wx (low AM) maize starch, yielding irregular fluorescence patterns, which may be due to the polyhedric granule shape. Wx maize starch granules are more irregular and rough than normal ones and a large proportion of these granules had severe internal cracking [38]. The patchiness is also pronounced for the SBS1 mutant but less so for the SBS2 mutant (Fig. 6), indicating that the SBS2 recognition motif is unevenly distributed on the granule surface. AMY1 bound to potato starch with low affinity as compared to the barley and maize starch granules and remarkably, mutation of either SBS eliminated adsorption to the surface of potato starch, while visible binding remained for barley and maize starch granules with these mutants. Despite the weaker affinity (Table 1), hydrolysis was observed and fluorescence was most intense in cavities in potato as well as barley starch granules. Thus, as for the other starches, SBSs are needed to interact with the granule surface, while the active site is sufficient for binding in the interior.

3.3. *Affinity gel electrophoresis (AGE)*

Further insight into the interaction between AMY1 and starch polysaccharides was gained by affinity gel electrophoresis with different polysaccharides included in the separation gel. Retarded migration of AMY1 wild-type (Fig. 7) indicated the strongest binding was to glycogen (relative migration 0.07), followed by pullulan (0.32), AP (0.70), with only weak binding to AM (0.93), suggesting α-1,6 linkages are important for recognition. For these polysaccharides, except AM, SBS1 or SBS2 mutants had increased mobility, reflecting reduced affinity and mutation of both SBSs showing migration almost at the same level as for the control gel. Remarkably, SBS2 mutation had the greatest impact, most prominently with pullulan for which the mobility ratio increased from 0.32 to 0.86 (0.69 for SBS1 mutant). Although these polysaccharides, except pullulan, are substrates for AMY1 and interact with the active site, the binding appears to be governed by the SBSs.

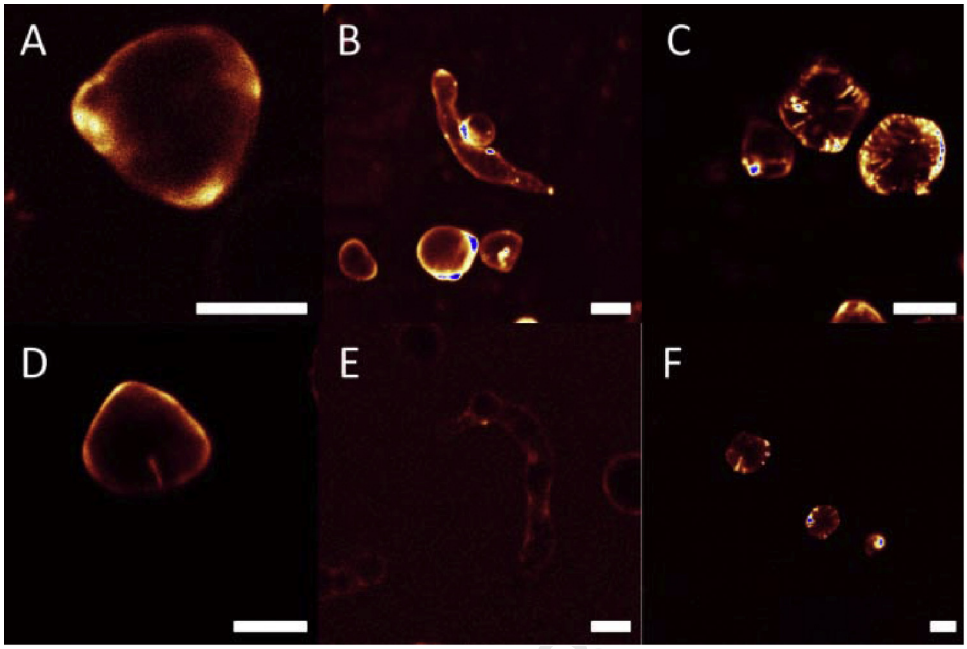

Fig. 6. Interaction between AMY1 SBS mutants and maize starch granules visualized by CLSM. Top (A–C) is the W278A/W279A (SBS1) mutant and bottom (D–F) is the Y380A (SBS2) mutant. Normal (A,D), High AM (B,E) and wx (low AM) (C,F) maize. See methods (Sections 2.5 and 2.6) for further details. The white calibration bars represent 10 μm.

3.4. Surface plasmon resonance (SPR)

Oligosaccharide binding properties of SBS1, SBS2, and the active site in AMY1 were analysed by measuring the affinity of each site using SPR (Table 2). In these experiments different forms of AMY1 were biotinylated in the presence of β-CD, which binds to both SBSs, to avoid obscuring the SBSs by the immobilisation on the streptavidin chip. β-CD does not protect the active site, which therefore may not have retained full integrity for oligosaccharide binding. In our experience with AMY1, this leads to a binding stoichiometry of significantly less than 1 at the active site which together with possible variation in how mutants orient during immobilization made it impossible to compare stoichiometries for the different proteins. However, stoichiometries for different ligands of a given mutant or AMY1 wild-type are regarded as safe to compare.

Firstly the affinity of the active site for linear oligosaccharides was determined by using an SBS1 mutant in the presence of 0.5 mM β-CD to block SBS2. By this approach the SBS1 mutant had K_d = 1.8 mM for maltoheptaose, matching K_M for this substrate of 1.2 mM [39], albeit at a somewhat different temperature (25 °C for K_d, 35 °C for K_M). In the absence of β-CD, K_d for maltoheptaose was 1.2 mM in the SBS1 mutant and notably the stoichiometry increased from 0.18 to 0.87, indicating binding to SBS2 with similar affinity as to the active site. K_d of maltoheptaose for wild-type was also 1.8 and 1.2 mM in the presence and absence of β-CD, respectively, with an accompanying increase in stoichiometry from 0.41 to 1.28, similar to that found for the SBS1 mutant (Table 2). However, the higher stoichiometry of wild-type AMY1 is unlikely to be due to binding of maltoheptaose to SBS1, as the SBS2 mutant, having an intact SBS1, exhibits lower binding stoichiometry for maltoheptaose than for β-CD which binds to SBS1. These findings suggest that binding of maltoheptaose to SBS1 is significantly weaker than at SBS2 or the active site and thus not discerned by using SPR, even though SBS1 was occupied along with SBS2 when soaking 10 mM maltoheptaose into crystals of the inactive catalytic nucleophile AMY1 D180A mutant [13]. Binding data of β-CD and maltoheptaose to AMY1 D180A indicate that using an active enzyme is not impacting the affinity, as K_d and stoichiometry were similar to wild-type AMY1 for these two oligosaccharides (Table 2). This leads to the conclusion that while maltoheptaose can bind to SBS1, the affinity is much weaker than at SBS2 or the active site.

AGE (Section 3.3) indicated branched polysaccharides may be particularly involved in the functional role of the SBSs. This motivated further investigation of AMY1 wild-type and SBS mutants by using α-limit dextrins of average DP 10 containing α-1,4 linked glucose with an α-1,6 branch, which revealed a K_d similar to maltoheptaose. Also similar to maltoheptaose data, α-limit dextrins showed lower stoichiometry than β-CD for the SBS2 mutant, while the opposite was true for the SBS1 mutant, strongly indicating that SBS2, but not SBS1 binds α-limit dextrin. Thus while β-CD is accommodated at both SBSs, but not the active site, linear and branched malto-oligosaccharides are found to primarily bind at the active site and SBS2, but not SBS1, indicating divergent roles for the SBSs.

4. Discussion

The surface of starch granules displays distinct structures dependent on plant genotype, anticipated to provide different interaction with enzymes. K_d for AMY1 with normal barley and maize starch was three- and ten-fold lower than with potato starch, respectively, suggesting AMY1 more readily binds to starch granule surfaces of A-type crystalline polymorph. The affinity towards the B-type polymorph pea starch was comparable to that of A-type, in agreement with C-type polymorph containing a mixture A- and B-types and with peripheral regions rich in A-type while the centre of pea starch granules is rich in B-type polymorph [40]. The tighter packing of the A-type polymorph could be critical for AMY1 binding if it interacts with multiple double helices at the two SBSs.

The wx (zero/low AM) starch differed little from normal starches in AMY1 binding, while the affinity was consistently lower for high AM starch, known to have a disturbed granule structure presumably hindering AMY1 binding. High AM maize has a smoother surface than normal maize starch granules and includes a subpopulation of granules with elongated, filamentous structures [19]. In addition, high AM as opposed to other maize starches is B-type polymorph and AP in high AM cereal starches has a relatively large proportion of very long chains similar to B-type polymorphs [19].

Table 2
AMY1 affinity and binding stoichiometry for oligosaccharides as determined by surface plasmon resonance.

Enzyme	β-Cyclodextrin		α-Limit dextrin		Maltoheptaose with β-CD		Maltoheptaose without β-CD	
	K_d (mM) ± SD	Stoich. ± SD	K_d (mM) ± SD	Stoich. ± SD	K_d (mM) ± SD	Stoich. ± SD	K_d (mM) ± SD	Stoich. ± SD
Wild-type[a]	0.09 ± 0.04 1.69 ± 0.20	1.76 ± 0.48	1.42 ± 0.37	0.94 ± 0.19	1.82 ± 0.48	0.41 ± 0.06	1.2	1.28
D180A	0.04 ± 0.01 1.40 ± 0.26	1.84 ± 0.11	n.d.	n.d.	n.d.	n.d.	0.75 ± 0.15	1.50 ± 0.15
W278A/W279A (SBS1)	0.07 ± 0.01	0.60 ± 0.13	3.49 ± 3.01	0.66 ± 0.13	1.76 ± 0.28	0.18 ± 0.01	1.15	0.87
Y380A (SBS2)	0.90 ± 0.05	0.93 ± 0.19	1.65 ± 0.66	0.57 ± 0.09	n.d.	n.d.	1.02 ± 0.59	0.40 ± 0.03

n.d. not determined.
[a] The interaction between wild-type AMY1 and β-cyclodextrin was fitted to a two-site binding model, however, for the other substrates the dissociation constants could not be resolved between the two sites as they were too close in affinity.

This confirms the importance of crystalline AP for AMY1 binding as low phosphate starch has lower AP content [21,31] and phosphate is an important modulator of AP crystallinity [30].

It is primarily the ends of AP double helices that are exposed at the granule surface and therefore vulnerable to amylases. As degradation of the granule progresses, cavities form where the amorphous regions as well as the sides of double helices of AP are exposed, both of which are subject to amylase catalysed hydrolysis [41,42]. Since AP double helices in both A (barley and maize) and B-type (potato) polymorphs are essentially identical with respect to helical structure [22,43], AMY1 binding in these cavities must be comparable between the A- and B-type polymorphs. Indeed, AMY1 binding is seen in such cavities for various starches, also when both SBSs were mutated or blocked by β-CD. Thus the active site is able to mediate binding to internal surfaces in starch granules.

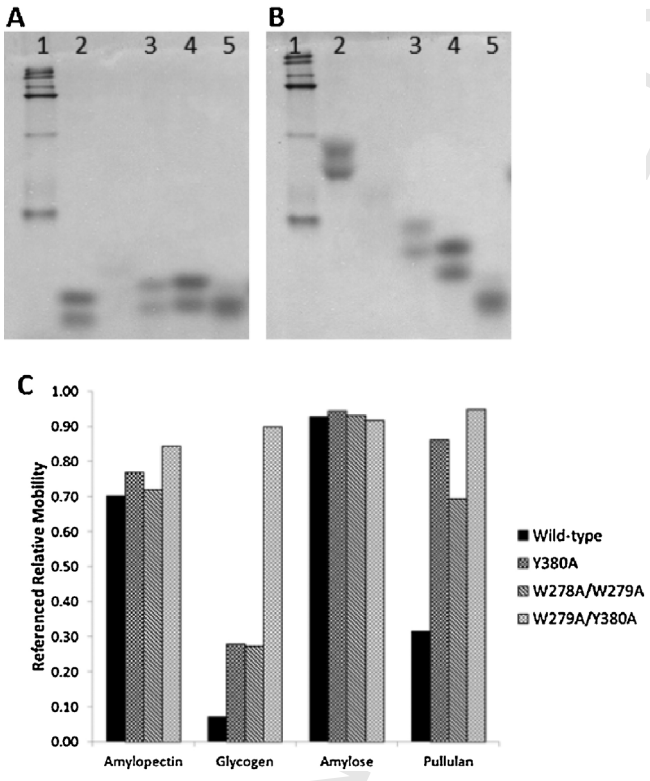

Fig. 7. Affinity electrophoresis of AMY1 and SBS mutants. (A) Control gel, (B) a gel containing 0.05% pullulan, (C) referenced relative mobility of AMY1 wild-type and SBS mutants for starch related polysaccharides. For each enzyme the ratio of the mobility in the polysaccharide containing gel to that in the control gel is divided by the same ratio for a non-interacting reference protein. This figure represents a single experiment, though the trends have been replicated under several different sets of conditions (varying voltage, run time, gel percentage). Wild-type (solid black, lane 2), Y380A (SBS2) mutant (checkerboard, lane 4), W278A/W279A (SBS1) mutant (diagonals, lane 3), the W279A/Y380A (SBS1/SBS2) mutant (crosshatched, lane 5). Lane 1 is the reference protein ladder.

SBSs are essential for AMY1 binding to the granule surface and AGE indicates that they also participate in binding to amorphous regions. In all cases SBS1 was more important than SBS2 for binding to granule surfaces, but less important in interaction with soluble polysaccharides as found by AGE. The importance of SBS2 was most pronounced with pullulan, which has the highest content of α-1,6 linkages, suggesting branch points or structures in starch depending on them constitute key SBS2 recognition motifs. Indeed progress curves for AMY1 hydrolysis of AP [18] revealed two distinguishable rates, the faster rate depending on SBS2. By contrast a single, SBS2 independent rate was observed for AM [18]. The current study showed binding of linear and branched malto-oligosaccharides to be mediated by SBS2 and the active site with very similar affinities. Such coordination of binding to the active site and an SBS was shown previously in two GH11 xylanases. Thus SPR analysis showed the active site and SBS of *Bacillus subtilis* xylanase having K_d for xylohexaose of 0.9 mM and 1.4 mM, respectively [44]. Additionally, the active site and SBS in *B. circulans* xylanase have similar K_d in the mM range for xylotetraose and xylohexaose as determined by NMR [45], but 10-fold higher k_{on} and k_{off} rates of the SBS suggested this is the initial point of xylan attachment. Notably, a few attempts to strengthen the affinity of SBSs were successful [46,47], but this did not enhance activity, perhaps because the correlation between SBS and active site affinity was disrupted which may decrease turn-over efficiency for instance if an SBS was continuing to hold on to a hydrolysis product that was too short to reach the active site.

The present results on starch granule binding support our previous findings that both SBS1 and SBS2 of AMY1 are critical for this interaction. We here provide further evidence that SBSs recognize different motifs on the granule surface, which in A-type polymorphs allows for synergistic interaction, perhaps due to the tighter packing of AP double helices. The resolution of CLSM was not sufficient to see differential binding of SBS mutants to the granule surface, though SBS1 appears to recognizes crystalline regions at the ends of AP double helices, perhaps a particular feature of blocklets [24], explaining its greater importance for binding to the granule surfaces and low affinity for malto-oligosaccharides. SBS2 by contrast is suited to recognize individual chains evidenced by its greater importance for binding in AGE and SPR experiments and by its role in patchy binding pattern in CLSM. Single chains may be present at the hair-like extensions from the blocklets found across the granule surface [24]. Particularly, it seems that α-1,6 linkages or regions of α-1,4 chains influenced by α-1,6 branch points are key in SBS2 binding. The higher affinity of SBS2 for β-CD supports that a more rigid form adopted by longer AP chains [22] would be a preferred target. The architecture of both SBSs seems well suited to these distinct roles as the platform created by the two tryptophans at SBS1 matches the curvature of AP double helices, while the "pair

of sugar tongs" formed by Tyr380 and His395 in SBS2 are positioned to accommodate an individual chain. This leads to a model where SBS1 is the initial site of AMY1 attachment onto the starch granule surface, while SBS2 plays a supporting role by binding a single chain near an α-1,6 branch point and in turn feeds a linear segment of the AP into the active site that is unable to accommodate branches near the point of hydrolysis. Once AMY1 penetrates the surface of the granule, SBS1 has a lesser (if any) role, while SBS2 continues to be important for isolating individual chains for the active site.

This study has provided new details on the individual roles played by the SBSs of AMY1, which is important not only for clarifying the mode of action of AMY1, but contributes to improving the understanding of the growing number of other enzymes identified as possessing SBSs. In most cases SBSs have only been studied superficially, making the few thoroughly examined examples crucial for our comprehension of these key features that provide distinct functional characteristics to polysaccharide converting enzymes.

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References

[1] E. Beck, P. Ziegler, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 (1989) 95–117.
[2] J.C. Rogers, C. Milliman, J. Biol. Chem. 258 (1983) 8169–8174.
[3] J.C. Rogers, J. Biol. Chem. 260 (1985) 3731–3738.
[4] D. Cockburn, B. Svensson, in: T.K. Lindhorst, A.P. Rauter (Eds.), Carbohydrate Chemistry: Chemical and Biological Approaches, Royal Society of Chemistry, Cambridge, 2013, pp. 204–221.
[5] S. Cuyvers, E. Dornez, J.A. Delcour, C.M. Courtin, Crit. Rev. Biotechnol. 32 (2012) 93–107.
[6] D. Guillén, S. Sánchez, R. Rodríguez-Sanoja, Appl. Microbiol. Biotechnol. 85 (2010) 1241–1249.
[7] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, Nucleic Acids Res. 42 (2014) D490–D495.
[8] B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, Nucleic Acids Res. 37 (2009) D233–D238.
[9] B. Svensson, T.G. Pedersen, I. Svendsen, T. Sakai, M. Ottesen, Carlsberg Res. Commun. 47 (1982) 55–70.
[10] N. Juge, J. Nøhr, M. Le Gal-Coëffet, B. Kramhøft, C.S.M. Furniss, V. Planchot, et al., Biochim. Biophys. Acta 1764 (2006) 275–284.
[11] A. Kadziola, M. Søgaard, B. Svensson, R. Haser, J. Mol. Biol. 278 (1998) 205–217.
[12] S. Tranier, K. Deville, X. Robert, S. Bozonnet, R. Haser, B. Svensson, et al., Biologia 60 (Suppl. 16) (2005) 37–46.
[13] X. Robert, R. Haser, H. Mori, B. Svensson, N. Aghajari, J. Biol. Chem. 280 (2005) 32968–32978.
[14] X. Robert, R. Haser, T. Gottschalk, F. Ratajczek, H. Driguez, B. Svensson, et al., Structure 11 (2003) 973–984.
[15] M.M. Nielsen, E. Seo, S. Bozonnet, N. Aghajari, X. Robert, R. Haser, et al., FEBS Lett. 582 (2008) 2567–2571.
[16] S. Bozonnet, M.T. Jensen, M.M. Nielsen, N. Aghajari, M.H. Jensen, B. Kramhøft, et al., FEBS J. 274 (2007) 5055–5067.
[17] M.M. Nielsen, S. Bozonnet, E. Seo, J.A. Mótyán, J.M. Andersen, A. Dilokpimol, et al., Biochemistry 48 (2009) 7686–7697.
[18] J.W. Nielsen, B. Kramhøft, S. Bozonnet, M. Abou Hachem, S.L.S. Stipp, B. Svensson, et al., Arch. Biochem. Biophys. 528 (2012) 1–6.
[19] M.A. Glaring, C.B. Koch, A. Blennow, Biomacromolecules 7 (2006) 2310–2320.
[20] I. Bocharnikova, L.A. Wasserman, A.V. Krivandin, J. Fornal, W. Baszczak, V.Y. Chernykh, et al., J. Therm. Anal. Cal. 74 (2003) 681–695.
[21] S.S. Kozlov, A. Blennow, A.V. Krivandin, V.P. Yuryev, Int. J. Biol. Macromol. 40 (2007) 449–460.
[22] I. Damager, S. Engelsen, A. Blennow, B.L. Møller, M.S. Motawia, Chem. Rev. 110 (2010) 2049–2080.
[23] S. Pérez, E. Bertoft, Starch - Stärke 62 (2010) 389–420.
[24] H. Park, S. Xu, K. Seetharaman, Carbohydr. Res. 346 (2011) 847–853.
[25] J. Jane, Z. Ao, S.A. Duvick, M. Wiklund, S. Yoo, K. Wong, et al., J. Appl. Glycosci. 50 (2003) 167–172.
[26] V. Planchot, P. Colonna, A. Buleon, Carbohydr. Res. 298 (1997) 319–326.
[27] V. Planchot, P. Colonna, D.J. Gallant, B. Bouchet, J. Cereal Sci. 21 (1995) 163–171.
[28] M. Carciofi, A. Blennow, S.L. Jensen, S.S. Shaik, A. Henriksen, A. Buléon, et al., BMC Plant Biol. 12 (2012) 223.
[29] A.K. Shrestha, J. Blazek, B.M. Flanagan, S. Dhital, O. Larroque, M.K. Morell, et al., Carbohydr. Polym. 90 (2012) 23–33.
[30] A. Blennow, S. Engelsen, Trends Plant Sci. 15 (2010) 236–240.
[31] A. Viksø-Nielsen, A. Blennow, K. Jørgensen, K.H. Kristensen, A. Jensen, B.L. Møller, Biomacromolecules 2 (2001) 836–843.
[32] A. Blennow, B. Wischmann, K. Houborg, T. Ahmt, K. Jørgensen, S. Engelsen, et al., Int. J. Biol. Macromol. 36 (2005) 159–168.
[33] H. Mori, K. Bak-Jensen, T.E. Gottschalk, M.S. Motawia, I. Damager, B.L. Møller, et al., FEBS J. 268 (2001) 6545–6558.
[34] N. Juge, J.S. Andersen, D. Tull, P. Roepstorff, B. Svensson, Protein Expr. Purif. 8 (1996) 204–214.
[35] P.J. Roberts, W.J. Whelan, Biochem. J. 76 (1960) 246–253.
[36] D.J. Gallant, B. Bouchet, A. Buleon, S. Perez, Eur. J. Clin. Nutr. 46 (1992) S3–S16.
[37] R.M. Gibson, B. Svensson, Carlsberg Res. Commun. 52 (1987) 373–379.
[38] A. Blennow, M. Hansen, A. Schulz, K. Jørgensen, A.M. Donald, J. Sanderson, J. Struct. Biol. 143 (2003) 229–241.
[39] A.W. MacGregor, J.E. Morgan, E.A. MacGregor, Carbohydr. Res. 227 (1992) 301–313.
[40] T.Y. Bogracheva, V.J. Morris, S.G. Ring, C.L. Hedley, Biopolymers 45 (1998) 323–332.
[41] G. Tawil, F. Jamme, M. Réfrégiers, A. Viksø-Nielsen, P. Colonna, A. Buléon, Anal. Chem. 83 (2011) 989–993.
[42] A. Pohu, J. Putaux, V. Planchot, P. Colonna, A. Buléon, Biomacromolecules 5 (2004) 119–125.
[43] R.F. Tester, J. Karkalas, X. Qi, J. Cereal Sci. 39 (2004) 151–165.
[44] S. Cuyvers, E. Dornez, M. Abou Hachem, B. Svensson, M. Hothorn, J. Chory, et al., Anal. Biochem. 420 (2012) 90–92.
[45] M.L. Ludwiczek, M. Heller, T. Kantner, L.P. McIntosh, J. Mol. Biol. 373 (2007) 337–354.
[46] S. Cuyvers, E. Dornez, M.N. Rezaei, A. Pollet, J.A. Delcour, C.M. Courtin, FEBS J. 278 (2011) 1098–1111.
[47] S. Cuyvers, E. Dornez, J.A. Delcour, C.M. Courtin, Appl. Microbiol. Biotechnol. 92 (2011) 539–549.