FEBS Letters 583 (2009) 1159–1163

FEBS Letters

journal homepage: www.FEBSLetters.org

A CBM20 low-affinity starch-binding domain from glucan, water dikinase

Camilla Christiansen [a,b], Maher Abou Hachem [b], Mikkel A. Glaring [a], Anders Viksø-Nielsen [c], Bent W. Sigurskjold [d], Birte Svensson [b,*], Andreas Blennow [a,*]

[a] VKR Research Centre, Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg, Denmark
[b] Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
[c] Novozymes A/S, DK-2880 Bagsvaerd, Denmark
[d] Department of Biology, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark

ARTICLE INFO

Article history:
Received 23 December 2008
Revised 11 February 2009
Accepted 27 February 2009
Available online 9 March 2009

Edited by Michael R. Sussman

Keywords:
Bioimaging
Carbohydrate-binding module 20
Glucan, water dikinase
Starch-binding domain
Surface plasmon resonance

ABSTRACT

The family 20 carbohydrate-binding module (CBM20) of the *Arabidopsis* starch phosphorylator glucan, water dikinase 3 (GWD3) was heterologously produced and its properties were compared to the CBM20 from a fungal glucoamylase (GA). The GWD3 CBM20 has 50-fold lower affinity for cyclodextrins than that from GA. Homology modelling identified possible structural elements responsible for this weak binding of the intracellular CBM20. Differential binding of fluorescein-labelled GWD3 and GA modules to starch granules *in vitro* was demonstrated by confocal laser scanning microscopy and yellow fluorescent protein-tagged GWD3 CBM20 expressed in tobacco confirmed binding to starch granules *in planta*.

1. Introduction

Enzymes binding reversibly to leaf starch granules are required during their synthesis or degradation as directed by photosynthesis. One recent example is the diurnal partitioning of the starch phosphorylator glucan, water dikinase 1 (GWD1) between the plastid stroma and the starch granule surface [1], highlighting redox-based regulation of starch metabolism during biosynthesis and degradation. The GWDs contain dedicated starch-binding domains (SBDs) (Fig. 1). Sequence-based classification gives SBDs in carbohydrate-binding module (CBM) families; 20, 21, 25, 26, 34, 41, 45, 48 and 53 [2] (http://www.cazy.org/fam/acc_CBM.html). CBM20 is encountered in archaea, bacteria, and eukaryota and occur mainly in α-amylases, β-amylases, cyclodextrin glucanosyltransferases, glucoamylases (GAs) and glucan, water dikinase (GWD).

SBDs having 90–130 amino acid residues are situated N- or C-terminally [3] and typically retain functionality in isolated form [4]. The structural fold of SBDs is conserved, consisting of a distorted β-barrel with 7–8 anti-parallel β-strands arranged in two β-sheets [5]. Most CBM20s possess two carbohydrate-binding sites providing bivalent interaction involving two or three conserved solvent accessible aromatic residues [6,7]. Site 1 is shallower and more solvent exposed than site 2 that undergoes significant structural changes upon binding [6]. CBM20s bind maltoheptaose and cyclodextrins [4,6] but their proposed main function is to attach to granular starch, thereby locally increasing the enzyme concentration at the substrate surface [8]. Another proposed role is to "unwind" α-glucan helices on the granule surface [9] resulting in a higher hydrolytic rate [10].

SBDs typically belong to extracellular amylolytic enzymes providing starch binding [7]. However, newly discovered families CBM45 [11] and CBM53 [12] in plant GWD and starch synthase III, respectively, have weak affinities for starch, suggesting that certain enzymes involved in intracellular starch metabolism in plants possess SBDs with new function. Recently, a putative CBM20 SBD was identified in partly starch granule bound GWD3 from *Arabidopsis thaliana* [13,14]. The properties of the corresponding recombinant GWD3-SBD are reported here and compared with CBM20 from *Aspergillus niger* GA [4], probing oligosaccharide binding by surface plasmon resonance (SPR) and binding to starch granules *in vitro* and *in vivo* by confocal laser scanning microscopy (CLSM). This first description of an isolated plant CBM20 demonstrates

Abbreviations: CBM, carbohydrate-binding module; CLSM, confocal laser scanning microscopy; GA, glucoamylase; GWD, glucan, water dikinase; SBD, starch-binding domain; SPR, surface plasmon resonance; YFP, yellow fluorescent protein

* Corresponding authors.
E-mail addresses: bis@bio.dtu.dk (B. Svensson), abl@life.ku.dk (A. Blennow).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.02.045

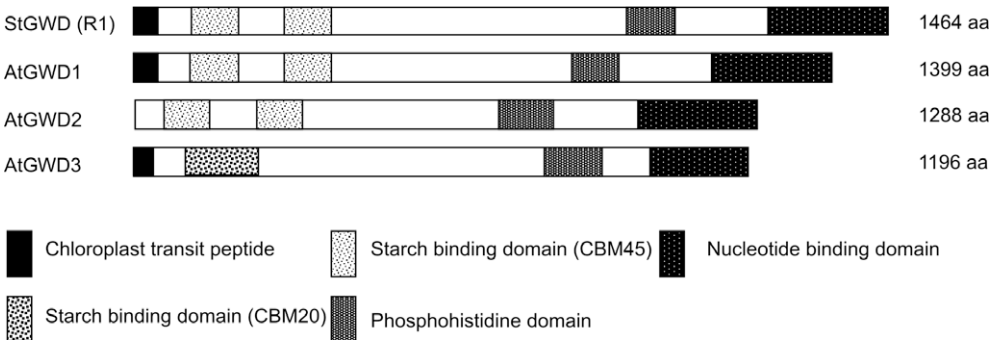

Fig. 1. Domain organisation of starch phosphorylating GWD homologues from *Arabidopsis thaliana* and *Solanum tuberosum*.

distinct function from classical fungal CBM20s, supposedly characteristic of intracellular non-hydrolytic enzymes involved in starch metabolism.

2. Materials and methods

2.1. Homology modelling and sequence alignment

A. niger GA-SBD (PDB 1KUL) was used as template (26% identity, 58% similarity for residues 509–616) to generate a GWD3-SBD homology model using Modeller v6.1 [15]. GWD3-SBD residues 170–184 lacking in the template were deleted from the model. Sequences were aligned using ClustalW.

2.2. Expression and purification of GWD3-SBD

GWD3-SBD (Asp^{68}–Gly^{184}) was cloned from full-length GWD3 cDNA [13] into NcoI and XhoI sites of pET-28a(+) (Novagen, Madison, WI), using sense: AACATGCCATGGCAGATGGATCAGGAACGAAAGTG (NcoI site underlined) and antisense primer: AACCGCTCGAGACCAACATCATCATCATTACCAAC (XhoI site underlined) and standard molecular biology procedures. *Escherichia coli* BL21(DE3) harbouring the GWD3-SBD construct was grown at 37 °C (LB medium; 50 μg/mL kanamycin) to OD_{600} ~0.5. Expression was induced by adding isopropyl β-D-thiogalactopyranoside to 250 μM, followed by incubation at 20 °C for 20 h. After cell harvest, sonication and centrifugation, the extract was passed over a 1 mL His-trap column (QIAGEN, Hamburg, Germany). The GWD3-SBD was eluted as recommended by the manufacturer, applied to a 6 mL β-cyclodextrin Sepharose column and eluted with 10 mM β-CD. β-CD traces were removed by an additional His-trap purification and desalting using 0.5 mL Zeba Desalt Spin Columns (Pierce, Rockford, IL). Final yield was ~0.7 mg/L culture. *A. niger* recombinant GA-SBD was produced as described [16].

2.3. Surface plasmon resonance (SPR) analysis

Surface plasmon resonance (Biacore T100; GE Healthcare, Uppsala, Sweden) sensorgrams were recorded of binding 0.01–10 mM (15 concentrations) α-, β-, γ-CD, linear maltooligosaccharides (DP 3–7) and β-CD with 2–6 phosphate groups (Sigma, Steinheim, Germany) to biotinylated protein (900–1000 RU on streptavidin-chip). K_d values were calculated by steady-state affinity fitting (BIAevaluation 1.1 software) to the response after subtracting the reference cell signal.

2.4. In vitro imaging of SBD binding

Fluorescein 5-EX succinimidyl ester (Invitrogen, Paisley, UK) in 4-fold molar excess was reacted with 100 μL GWD3-SBD (1 mg/mL, 50 mM HEPES-carbonate, pH 8.0, 10 mM β-CD) (final 140 μL) for 1 h at 20 °C. Fluorescein-conjugated protein was His-trap purified, desalted twice and 10 μM protein was incubated with starch granules (10 mg/mL) for 45 min at 4 °C with gentle rotation. Visualisation by CLSM (TCS SP2, Leica Microsystems, Germany) [17] used fluorescein excitation at 488 nm and recorded emission at 500–550 nm with 25% laser power and the gain varied to prevent saturation of the detector and to ensure comparable intensities. The objective used was HCX PL APO 63.0×1.20 W CORR UV. Image analysis was performed by the TCS SP2 software.

2.5. Transient expression of GWD3-SBD-YFP

The region encoding residues 1–201 of GWD3, containing the chloroplast transit peptide and SBD, was amplified using uracil-containing primers (sense: GGCTTAAUATGGAGAGCATTGGCAGCCATTG, antisense: GGTTTAAUCCCACTACTCTATCATCACCAACATCA) and fused to YFP [18]. The fusion protein was transiently expressed in *Nicotiana benthamiana* and analysed in situ by CLSM [18].

3. Results and discussion

3.1. Sequence analysis and homology modelling of the GWD3-SBD

The sequence of *A. thaliana* CBM20 has 26% identity/58% similarity to *A. niger* GA-SBD compared to 50% identity/69% similarity with a putative GWD3-SBD from *Oryza sativa*. Tryptophans at GWD3-SBD putative binding site 1 (W102 and W142) are conserved as are W115 (putative binding site 2) and W164 (structural in GA-SBD) (Fig. 2). The GWD3-SBD model exhibited a similar fold as GA-SBD with seven β-strands distributed in two β-sheets, and ligand-binding site 1 was readily identified (Fig. 3A). Only F86 and W115 are conserved in GWD3-SBD from site 2, containing Y527, Y556 and W563 in GA-SBD. Noticeably, GWD3-SBD has a 7 residue deletion in the third, flexible GA-SBD loop including Y556 that is strongly involved in binding at site 2 (Fig. 2). The GWD3-SBD model (Fig. 3B) illustrated this structural difference presumably affecting the binding.

3.2. Purification and characterisation of GWD3-SBD

A GWD3-SBD construct containing residues 68–184 was the most stable one among those tested (not shown). The purified module had an apparent molecular weight in SDS–PAGE consistent with the calculated 14.2 kDa (Fig. 4). Nonetheless, the stability of GWD3-SBD was lower (T_m = 44.6 °C, Supplementary data) than that of GA-SBD (T_m = 56.7 °C) [19]. Stability was lower after re-purification for efficient removal of β-CD indicative of ligand-stabilisation. It is also possible that interactions with the catalytic

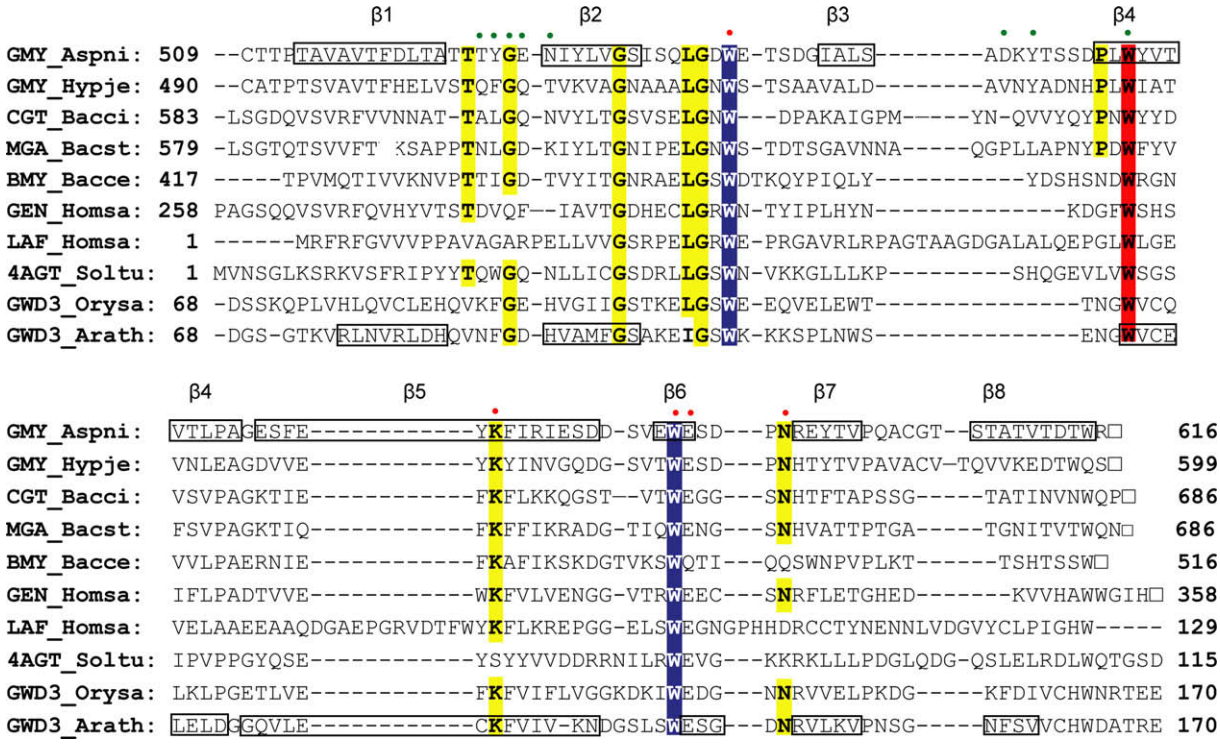

Fig. 2. Alignment of bacterial and eukaryotic CBM20s. Highly conserved binding site 1 and 2 tryptophans are highlighted in blue and red, respectively, other conserved residues are in yellow. Binding residues in site 1 and 2 are marked by red and green dots, respectively. β-Strands of GA-SBD and the GWD3-SBD model are boxed. GA: glucoamylase, CGT: cyclodextrin glucanosyl transferase, MGA: maltogenic α-amylase, BMY: β-amylase, GEN: genethonin-1, LAF: laforin, 4AGT: 4-α-glucanotransferase, GWD3: glucan, water dikinase 3.

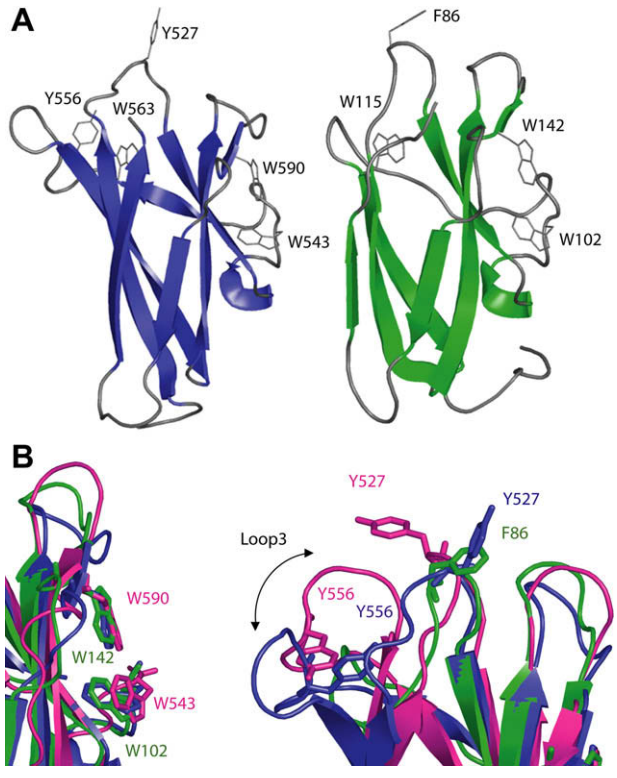

Fig. 3. (A) Structures of GA-SBD (PDB 1KUL, blue) and *A. thaliana* GWD3-SBD model (template 1KUL, green). (B) Superimposition of binding site 1 (left) and 2 (right) of β-CD (not included for clarity) GA-SBD complex (magenta), free GA-SBD (blue), and the GWD3-SBD model (green).

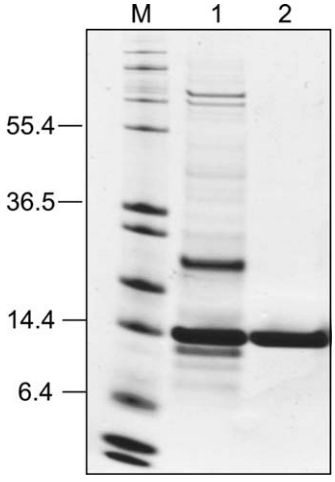

Fig. 4. SDS–PAGE of recombinant GWD3-SBD. Lanes M: Marker (kDa), 1: GWD3-SBD after His-trap elution, 2: GWD3-SBD after β-CD-Sepharose.

GWD3 domain provide stability to SBD when present in the native enzyme.

3.3. Affinity to cyclodextrins as determined by SPR

Differential affinity of GWD3-SBD and GA-SBD towards α-, β-, and γ-CD was demonstrated using SPR (Table 1). GWD3-SBD showed strongest binding towards α-CD, and no detectable affinity for linear maltooligosaccharides (DP 3–7) or for phosphorylated β-CD. The β-CD affinity minimum was at neutral pH. GWD3-SBD

Table 1
Dissociation constants for GWD3-SBD and GA-SBD determined using SPR.

CBM20	Buffer	Ligand	K_d (mM)
GWD3-SBD	pH 5.0	β-CD	0.62 ± 0.02
	pH 5.5	α-CD	0.22 ± 0.01
		β-CD	0.38 ± 0.07
		γ-CD	0.84 ± 0.66
	pH 6.0	β-CD	0.94 ± 0.02
	pH 6.5	β-CD	1.09 ± 0.08
	pH 7.0	α-CD	0.45 ± 0.01
		β-CD	1.07 ± 0.19
		γ-CD	1.47 ± 0.40
	pH 9.0	α-CD	0.59 ± 0.16
		β-CD	0.56 ± 0.12
		γ-CD	5.56 ± 2.07
GA-SBD	pH 5.5	β-CD	$7.5 \pm 1.30 \times 10^{-3}$

Samples were analysed in duplicates.

forms expressed in *Pichia pastoris* and as a glutathione *S*-transferase (GST) fusion in *Escherischia coli* (not shown) gave similar K_d values for β-CD at pH 7.0, excluding artefacts due to expression host or affinity tag. The optimum affinity for β-CD of *A. niger* GA is at pH 4.5 [20] and comparison at the GWD3-SBD optimum pH 5.5 showed affinity 50-fold higher for GA- than for GWD3-SBD (Table 1) – consistent with GA UV difference spectroscopy (K_d = 14.4 μM [21]) and NMR titration (K_d = 9 μM [22]). Affinity differences between the two GA-SBD sites assessed for the mutants, W563K (site 2) and W590K (site 1) [21] are reflected in K_d of 28 μM and 6.4 μM, respectively. This agrees with K_{d1} of 2.0 and K_{d2} of 31.3 μM obtained by fitting a two binding site model to our GA-SBD SPR data (not shown).

The 50-fold higher K_d for β-CD (Table 1) clearly identifies GWD3-SBD as a low-affinity SBD compared to GA-SBD. The physiological relevance and generality is substantiated by the plant CBM45 in GWD1 [11] and CBM53 in starch synthase III [12] both having low affinity. As for GWD3 these enzymes are predominantly found in soluble form when extracted. Furthermore, GWD1 binding to starch *in planta* is stronger in the dark [1,23] as a possible effect of the redox potential in stroma [1]. However, GWD3 does not possess the suggested CFATC redox motif found in GWD1 and might not be redox regulated. The slightly lower affinity found at pH 6.5 as compared to pH 9 may suggest slightly stronger binding during the day when stromal pH increases indicating differential dynamics of binding for the GWD3-SBD in response to physiological needs.

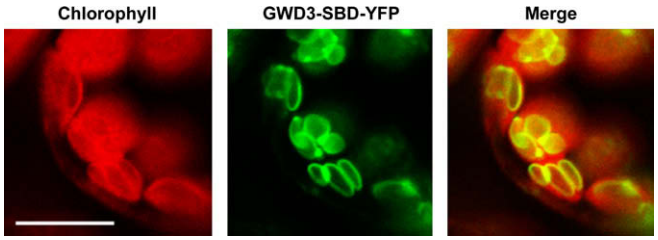

Fig. 6. GWD3-SBD-YFP transiently expressed with its *A. thaliana* GWD3 transit peptide in tobacco leaf mesophyll cells, showing binding at the surface of starch granules. Scale bar: 10 μm.

3.4. Binding of GWD3-SBD to starch granules in vitro and in vivo

CLSM visualised binding of fluorescein-labelled GWD3-SBD to starch granules, including normal and waxy (high amylopectin content) barley and maize endosperm starch (type A) and normal potato tuber starch (type B). Clear fluorescence was detected at the granule surface (Fig. 5) and in channels – characteristic of maize starches [24]. The sites of label in the starch granules indicate identical binding sites for the two SBDs. However, some subtle differences in binding distribution to specific starches were observed. While both SBDs interacted internally with the waxy granules, they showed more discrete surface binding to normal cereal granules. The potato starch showed least penetration. For the maize granules the label for the GA-SBD had higher contrast than the GWD3-SBD indicating a tendency for more distinct surface localisation for GA-SBD than for GWD3-SBD suggesting that GWD3-SBD more efficiently penetrates these granules. Fluorescein labelled bovine serum albumin gave no starch granule fluorescence and free dye penetrated the granules and resulted in unspecific binding (Supplementary data). *In vivo*, a GWD3-SBD-YFP fusion demonstrated affinity for starch granules by transient expression in tobacco leaves (Fig. 6). YFP fluorescence was localised in the chloroplasts (red chlorophyll autofluorescence) and was essentially confined to the starch granules.

4. Conclusion

The present functional characterisation of an isolated plant CBM20 provides evidence for *in vitro* and *in vivo* binding of a group of intracellular CBM20s with substantially lower and therefore

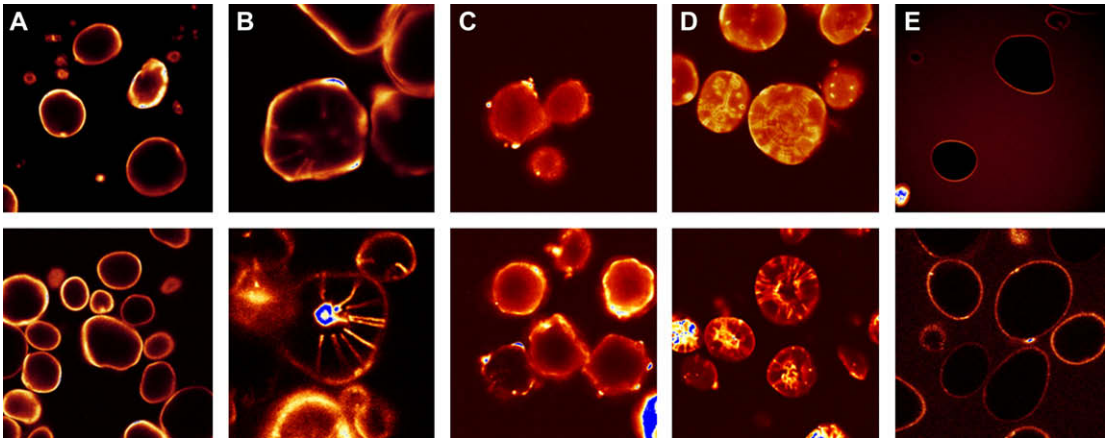

Fig. 5. Interaction of fluorescein-labelled GWD3-SBD (top panels) and GA-SBD (bottom panels) with different starches: (A) normal barley; (B) normal maize; (C) waxy barley; (D) waxy maize; and (E) potato.

potentially more dynamic affinity for starch granules than CBM20s from microbial hydrolases. Structural difference between GWD3- and GA-SBD in a flexible loop at binding site 2 probably plays a role in the low affinity of GWD3-SBD.

Acknowledgements

This work was supported by the Danish Natural Science Research Council, the Danish Research Council for Technology and Production Sciences (M.A.H., M.A.G.), the Carlsberg Foundation, and a FOBI PhD stipend (C.C.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.02.045.

References

[1] Mikkelsen, R., Mutenda, K.E., Mant, A., Schurmann, P. and Blennow, A. (2005) A-glucan, water dikinase (GWD): a plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity. Proc. Natl. Acad. Sci. USA 102, 1785–1790.
[2] Machovic, M. and Janecek, S. (2006) Starch-binding domains in the post-genome era. Cell Mol. Life Sci. 63, 2710–2724.
[3] Svensson, B., Jespersen, H., Sierks, M.R. and MacGregor, E.A. (1989) Sequence homology between putative raw-starch binding domains from different starch-degrading enzymes. Biochem. J. 264, 309–311.
[4] Giardina, T., Gunning, A.P., Juge, N., Faulds, C.B., Furniss, C.S.M., Svensson, B., Morris, V.J. and Williamson, G. (2001) Both binding sites of the starch-binding domain of *Aspergillus niger* glucoamylase are essential for inducing a conformational change in amylose. J. Mol. Biol. 313, 1149–1159.
[5] Sorimachi, K., Jacks, A.J., Le Gal-Coëffet, M.F., Williamson, G., Archer, D.B. and Williamson, M.P. (1996) Solution structure of the granular starch binding domain of glucoamylase from *Aspergillus niger* by nuclear magnetic resonance spectroscopy. J. Mol. Biol. 259, 970–987.
[6] Sorimachi, K., Le Gal-Coëffet, M.F., Williamson, G., Archer, D.B. and Williamson, M.P. (1997) Solution structure of the granular starch binding domain of *Aspergillus niger* glucoamylase bound to β-cyclodextrin. Structure 5, 647–661.
[7] Penninga, D., van der Veen, B.A., Knegtel, R.M.A., van Hijum, S.A.F.T., Rozeboom, H.J., Kalk, K.H., Dijkstra, B.W. and Dijkhuizen, L. (1996) The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. J. Biol. Chem. 271, 32777–32784.
[8] Boraston, A.B., Bolam, D.N., Gilbert, H.J. and Davies, G.J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem. J. 382, 769–781.
[9] Morris, V.J., Gunning, A.P., Faulds, C.B., Williamson, G. and Svensson, B. (2005) AFM images of complexes between amylose and *Aspergillus niger* glucoamylase mutants, native, and mutant starch binding domains: a model for the action of glucoamylase. Starch-Starke 57, 1–7.
[10] Southall, S.M., Simpson, P.J., Gilbert, H.J., Williamson, G. and Williamson, M.P. (1999) The starch-binding domain from glucoamylase disrupts the structure of starch. FEBS Lett. 447, 58–60.
[11] Mikkelsen, R., Suszkiewicz, K. and Blennow, A. (2006) A novel type carbohydrate-binding module identified in α-glucan, water dikinases is specific for regulated plastidial starch metabolism. Biochemistry 45, 4674–4682.
[12] Valdez, H.A., Busi, M.V., Wayllace, N.Z., Parisi, G., Ugalde, R.A. and Gomez-Casati, D.F. (2008) Role of the N-terminal starch-binding domains in the kinetic properties of starch synthase III from *Arabidopsis thaliana*. Biochemistry 47, 3026–3032.
[13] Baunsgaard, L., Lütken, H., Mikkelsen, R., Glaring, M.A., Pham, T.T. and Blennow, A. (2005) A novel isoform of glucan, water dikinase phosphorylates pre-phosphorylated α-glucans and is involved in starch degradation in *Arabidopsis*. Plant J. 41, 595–605.
[14] Kötting, O., Pusch, K., Tiessen, A., Geigenberger, P., Steup, M. and Ritte, G. (2005) Identification of a novel enzyme required for starch metabolism in Arabidopsis leave. The phosphoglucan, water dikinases. Plant Physiol. 137, 242–252.
[15] Sali, A. and Blundell, T.L. (1993) Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815.
[16] Christensen, T., Woeldike, H., Boel, E., Mortensen, S.B., Hjortshøj, K., Thim, L. and Hansen, M.T. (1988) High level expression of recombinant genes in *Aspergillus oryzae*. Bio. Technol. 6, 1419–1422.
[17] Blennow, A., Hansen, M., Schulz, A., Jorgensen, K., Donald, A.M. and Sanderson, J. (2003) The molecular deposition of transgenically modified starch in the starch granule as imaged by functional microscopy. J. Struct. Biol. 143, 229–241.
[18] Glaring, M.A., Zygadlo, A., Thorneycroft, D., Schulz, A., Smith, S.M., Blennow, A. and Baunsgaard, L. (2007) An extra-plastidial α-glucan, water dikinase from *Arabidopsis* phosphorylates amylopectin in vitro and is not necessary for transient starch degradation. J. Exp. Bot. 58, 3949–3960.
[19] Christensen, T., Svensson, B. and Sigurskjold, B.W. (1999) Thermodynamics of reversible and irreversible unfolding and domain interactions of glucoamylase from *Aspergillus niger* studied by differential scanning and isothermal titration calorimetry. Biochemistry 38, 6300–6310.
[20] Goto, M., Tanigawa, K., Kanlyakrit, W. and Hayashida, S. (1994) The mechanism of binding of glucoamylase 1 from *Aspergillus-awamori* var *Kawachi* to cyclodextrins and raw starch. Biosci. Biotechnol. Biochem. 58, 49–54.
[21] Williamson, M.P., Le Gal-Coëffet, M.F., Sorimachi, K., Furniss, C.S., Archer, D.B. and Williamson, G. (1997) Function of conserved tryptophans in the *Aspergillus niger* glucoamylase 1 starch binding domain. Biochemistry 36, 7535–7539.
[22] Le Gal-Coëffet, M.F., Jacks, A.J., Sorimachi, K., Williamson, M.P., Williamson, G. and Archer, D.B. (1995) Expression in *Aspergillus niger* of the starch-binding domain of glucoamylase. Comparison with the proteolytically produced starch-binding domain. Eur. J. Biochem. 233, 561–567.
[23] Ritte, G., Lorberth, R. and Steup, M. (2000) Reversible binding of the starch-related R1 protein to the surface of transitory starch granules. Plant J. 21, 387–391.
[24] Glaring, M.A., Koch, C.B. and Blennow, A. (2006) Genotype-specific spatial distribution of starch molecules in the starch granule: a combined CLSM and SEM approach. Biomacromolecules 7, 2310–2320.