

Production and purification of a granular-starch-binding domain of glucoamylase 1 from *Aspergillus niger*

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A domain of glucoamylase 1 from *Aspergillus niger* which binds to granular starch was produced by proteolytic digestion and purified to apparent homogeneity by extraction with corn starch followed by anion-exchange chromatography and gel filtration. The peptide has a molecular weight of 25 100, contains approximately 38% carbohydrate (w/w) and corresponds to residues 471–616 at the C-terminus of glucoamylase 1. The peptide bound to granular corn starch maximally at 1.08 nmol/mg starch. It inhibited the hydrolysis of granular starch by glucoamylase 1 but had no effect on the hydrolysis of starch in solution.

Glucoamylase; Binding domain; Starch binding; Proteolysis; *Aspergillus niger*

1. INTRODUCTION

Previous studies on the structures of glucoamylases (1,4- α -D-glucan glucohydrolase EC 3.2.1.3) from a variety of microbial sources have shown that the isoenzyme which does not bind to granular starch (G2) is generated in vivo by the post-translational modification of G1 [1–3]. Both enzymes are active towards soluble substrates but G1 shows increased activity towards insoluble forms. This led to the proposal that G1 contains two separate domains: a catalytic domain containing the active site, and a granular starch binding domain that confers on the enzyme the ability to be absorbed onto the surface of granular starch [4]. G1 contains about 100 more amino acids at the C-terminus than G2 [1] and so this region was suggested to be the domain enabling binding to granular starch. Sequence homology data has recently shown that similar domains are present in many granular-starch-digesting enzymes, including α -amylase from *Streptomyces limosus*, β -amylase from *Clostridium thermosulfurogenes* and cyclodextrin glucanotransferase from *Klebsiella pneumoniae* [5].

The putative granular-binding domain is separated from the catalytic domain by a region of about 40 amino acids, rich in glycosylated serine and threonine residues, that may stabilise the enzyme and protect against proteolytic attack [6]. However, studies on a peptide obtained from the glycosylated region from *Aspergillus awamori* var. *kawachi* showed that the pep-

tide bound to granular starch and, in competition experiments with G1, promoted granular starch hydrolysis [7].

As a first step towards resolving the function of the granular starch binding domain and the Ser- and Thr-rich region, and their interaction with the catalytic domain, we report on the production, purification and properties of a peptide from *Aspergillus niger* G1, containing both the granular starch binding domain and the Ser- and Thr-rich region.

2. METHODS

2.1. Materials

Aspergillus niger glucoamylase (6100 U/ml), *Bacillus licheniformis* protease type VIII (11.3 U/mg), granular corn starch and soluble potato starch were from Sigma Chemical Co. All reagents were of analytical grade.

2.2. Proteolytic digestion of glucoamylase

Glucoamylase (9.5 g) was precipitated from a crude preparation containing both G1 and G2 by fractionation with 80% ammonium sulphate. The precipitate was collected by centrifugation at $20\,000 \times g$ for 30 min, redissolved in 6.5 mM citric acid/90 mM sodium phosphate pH 7.0 and dialysed extensively against the buffer. The dialysed solution was diluted to 1 liter with buffer and protease VIII (65 mg) added. Incubation was at 30°C with stirring and was monitored by anion-exchange chromatography on aliquots removed at time intervals. The reaction was terminated after 24 h by adjusting the pH to 3.6 with HCl and cooling to 2°C.

2.3. Isolation of starch binding components

G1C was extracted from the digestion mixture by adding about 50 g of granular corn starch and stirring at 2°C for 4 h. The starch was sedimented by centrifugation at $10\,000 \times g$ for 20 min, washed with 5 mM sodium acetate buffer pH 3.6 and centrifuged. The supernatants were pooled and re-extracted twice more with granular starch. Bound protein was eluted from the starch by 200 ml 0.05 M sodium borate pH 8.2 with stirring at room temperature for 2 h. Starch was removed by centrifugation and re-extracted twice more with borate. The borate extracted supernatants were pooled, concentrated to

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Abbreviations: G1, glucoamylase 1; G2, glucoamylase 2; G1C, C-terminal granular-starch-binding domain from glucoamylase 1 (residues 471–616)

about 50 ml by rotary evaporation under vacuum at 40°C, and dialysed in benzoylated dialysis tubing (MW cut-off 2000 from Sigma Chemical Co.) against 2 mM Tris/HCl buffer pH 7.0. This was designated crude G1C.

2.4. Anion-exchange chromatography

A Mono Q(HR10/10) column (Pharmacia LKB Biotechnology, Milton Keynes, United Kingdom) was equilibrated in 2 mM Tris/HCl buffer pH 7.0. Crude G1C (1 ml) was applied to the column at a flow-rate of 2 ml/min, and eluted with a 0.35–0.5 M gradient of KCl. 1 ml fractions were collected. The run was repeated 45 times and the fractions pooled, lyophilised, redissolved in water and dialysed in benzoylated dialysis tubing against water.

2.5. Size exclusion chromatography

A Sephacryl S-200 column (25 × 900 mm) in 50 mM sodium acetate pH 5.0:1 M NaCl was run at a flow rate of 0.5 ml/min for preparative chromatography. For analytical chromatography, a Tosoh G3000SW column (Anachem Ltd, UK) was equilibrated with 6M guanidine hydrochloride/0.1 mM DTT. Proteins dissolved in mobile phase (20 µl) were injected onto the column at a flow-rate of 0.5 ml/min. The column was calibrated with blue dextran, bovine serum albumin, ovalbumin, trypsinogen and α-lactalbumin.

2.6. Purification of G1

Glucoamylase 1 for binding experiments was purified as described by Subbaramaiah and Sharma [8].

2.7. Assays

Protein concentrations were determined according to Lowry et al. [9]. Soluble starch hydrolysing activity was determined according to the method described by Rick and Stegbauer [10]. One unit of activity is the amount of enzyme that liberates 1 µmol of glucose per ml per min at pH 6.9 and 25°C. The carbohydrate content of G1C was determined by the anthrone-sulphuric acid method with mannose as standard [11]. The amino acid composition of G1C was determined by Cambridge Research Biochemicals Ltd. after bound carbohydrate had been removed by β-elimination under reducing conditions [3]. The N-terminal sequence was determined by the DABITC method [12]. SDS-polyacrylamide gel electrophoresis was performed on a 10% gel according to Laemmli [13] and proteins visualised by silver staining. Binding data was analysed by the method of Wilkinson [14].

3. RESULTS

3.1. Production and purification of G1C

The proteolytic digestion of G1 was monitored by anion-exchange chromatography on aliquots removed during the reaction. Fig. 1A shows two peaks in the crude glucoamylase which are G2 and G1 respectively. It can be seen from Fig. 1B after 24 h digestion that G1 has almost completely been digested, with the subsequent generation of G1C at 21 ml together with an analogue of G2, which elutes close to the original G2 peak. After extraction with granular corn starch, crude G1C was chromatographed on an anion-exchange column. Fractions from the column were assayed for protein and for activity on soluble starch (Fig. 2). The peak fractions at 22 ml, containing protein but with little activity on soluble starch, were pooled. Gel filtration of this pooled sample on Sephacryl S200 removed residual glucoamylase from G1C. The yield of peptide was 55 mg, and G1C exhibited no hydrolytic activity on soluble or granular starch.

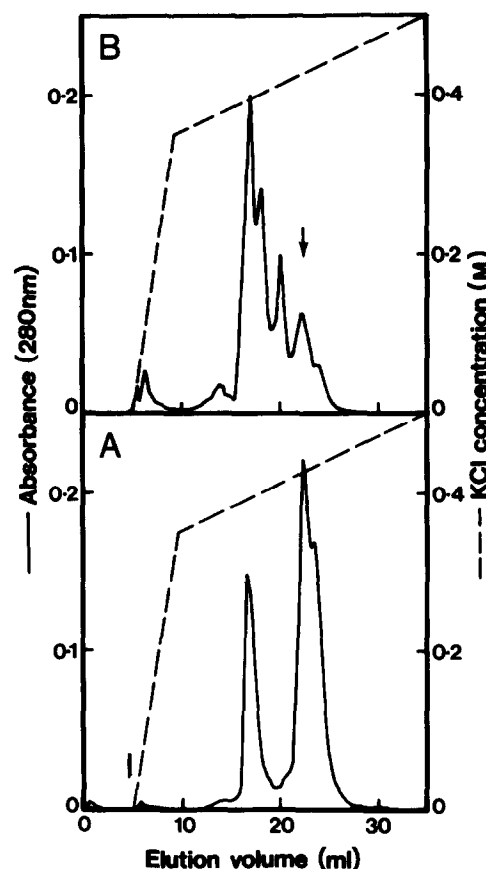


Fig. 1. Anion-exchange chromatography of the protease digestion of glucoamylase at the start of the reaction (A) and after 24 h (B). The arrow indicates the position of G1C.

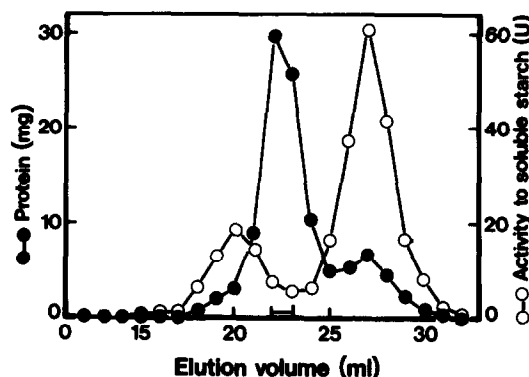


Fig. 2. Protein concentration (●) and soluble starch hydrolysing activity (○) for the fractions from anion-exchange chromatography of G1C. The bar indicates the fractions which were pooled and designated G1C.

3.2. Characterisation of G1C

G1C gave 1 sharp, symmetrical peak on size exclusion chromatography with a molecular mass of $25\,100 \pm 200$ Da (5 determinations). On SDS-PAGE, G1C was also apparently homogeneous, but gave a broad, light brown band after silver staining, typical of glycoproteins. Consequently, the molecular weight

could not be reliably estimated by SDS-PAGE. The carbohydrate content was 38% (w/w), corresponding to 56 mol mannose/mol G1C. The amino acid composition is given in Table I, and the N-terminal sequence was A-X-G-G which corresponds to residues 471–474 of G1 [15] where X is a glycosylated threonine residue. From these data, G1C consists of residues 471–616 on the original G1 molecule.

From the amino acid composition and the extent of glycosylation [16], the molecular weight of G1C was calculated to be 24 886 Da, which is in good agreement with the figure obtained from size exclusion chromatography. Using the amino acid composition, the theoretical absorption coefficient ($\epsilon_{280}^{\text{theor}}$) was calculated [17] to be $30.7 \text{ mM}^{-1} \text{ cm}^{-1}$. This value was used to calibrate the protein assay.

3.3. Binding properties of G1C

G1C bound to granular starch (Fig. 3). The amount of protein bound depended on the initial protein concentration in the assay. A Michaelis/Langmuir-type equation was used to relate the extent of protein adsorbed, q_{ad} , to the free protein in solution at equilibrium, q :

$$q_{\text{ad}}/q = K_p \cdot q_{\text{max}}/(1 + K_p \cdot q)$$

where K_p is a constant and q_{max} is the maximum amount of protein adsorbed by the substrate [18]. For G1C, $q_{\text{max}} = 1.08 \pm 0.02 \text{ nmol G1C/mg starch}$ and $K_p = 12.7 \pm 0.5 \mu\text{M}$. For G1, q_{max} was lower ($0.26 \pm 0.01 \text{ nmol G1/mg starch}$) with $K_p = 2.6 \pm 0.2 \mu\text{M}$. The differences in binding reflect the smaller size of G1C com-

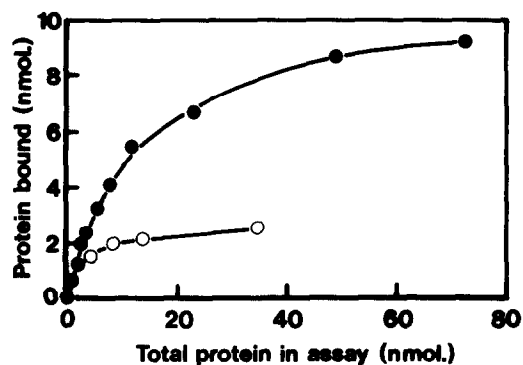


Fig. 3. Adsorption isotherms for G1 (○) and G1C (●) to granular corn starch. Protein (0–3 mg/ml) was incubated with granular corn starch (10 mg/ml) in 5 mM sodium acetate buffer pH 3.6 at 4°C in a rotating incubator (20 rpm) for 16 h. The reaction was terminated by centrifugation in an MSE microfuge for 5 min. The protein concentration in the supernatant was determined and subtracted from the total protein to give amount of protein bound.

pared to the parent protein, and to the additional presence of the catalytic domain in G1 which may partly contribute to the association.

G1C competes with G1 for the binding of granular starch. Fig. 4 shows that increasing concentrations of G1C lead to decreasing hydrolytic activity of G1 towards granular starch. For example, a G1C concentration of $0.65 q_{\text{max}}$ (65% saturation) leads to a 93% decrease in the activity of G1. G1C had no effect on the hydrolysis of soluble starch by G1 (results not shown).

4. DISCUSSION

Conflicting evidence has been reported on the nature and location of the granular-starch-binding domain

Table I

Amino acid composition of G1C		
Amino acid	Expected ¹	Obtained
Phe	3	3
Pro	6	7
Gly	10	10
Leu	6	6
Glx	10	11
Asx	11	12
His	0	0
Met	0	0
Arg	3	3
Tyr	6	6
Lys	4	2
Ile	5	3
Trp	4	ND
Cys	2	ND
Ala	12	18 ²
Thr	15 ³	12
Ser	11 ³	11
Val	9	6

1, The expected value is from residues 471–616 of G1. 2, Ala is overestimated due to inference from two non-standard peaks. 3, These values are the number of non-glycosylated residues. Thr and Ser that were glycosylated in the original sample gave two non-standard peaks in the chromatogram. ND, not determined.

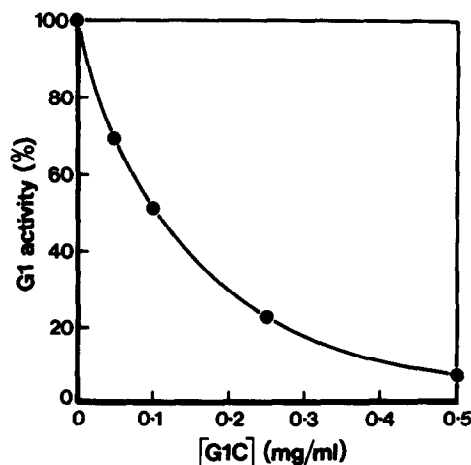


Fig. 4. Effect of [G1C] on the hydrolysis of granular starch by G1. G1 (0.1 mg/ml) was incubated together with granular corn starch (10 mg/ml) and G1C (0–0.5 mg/ml) in 5 mM sodium acetate buffer pH 3.6 at 25°C in a rotating incubator (20 rpm) for 24 h. The reaction was terminated by centrifugation in an MSE microfuge for 5 min. Hydrolysis was determined by measuring the concentration of reducing groups in the supernatant. 100% hydrolysis is equivalent to 1.05 nmol/min.

within the G1 molecule. Since G2 does not contain residues 514–616, this region was reported to be the granular-starch-binding domain and was shown to have homology with other putative granular-starch-binding domains from a variety of granular-starch-digesting enzymes from a range of organisms [5]. Also, a fusion protein of *E.coli* α -galactosidase and residues 484–616 from *A. niger* G1 bound to granular starch [19]. The highly glycosylated serine and threonine rich region consisting of residues 471–513 on G1 was thought to stabilise the domain against proteolytic attack and play no role in the interaction of G1 with granular starch [6]. However, data obtained with this isolated region from the highly homologous glucoamylase 1 from *A. awamori* var. *kawachi* suggested that this was the granular-starch affinity site since, after proteolysis, this region not only bound to granular starch but promoted its digestion with glucoamylase 1. The deglycosylation of this peptide resulted in the loss of granular starch binding capacity [7]. An analogous structure was reported for fungal cellobiohydrolases in which the catalytic domain and the binding domain are connected by a highly glycosylated region rich in serine, threonine and proline. Recent evidence suggests that this interconnecting region is not required for the binding domain to interact with insoluble substrates [20].

The only way to resolve the fraction of the C-term domains in amylases is by purification of a functional binding domain. This report shows that after proteolysis, residues 471–616 of G1 contain a functional granular-starch-binding domain, but whether the minimum domain is contained in residues 471–513 (region A) or in residues 514–616 (region B) remains unresolved. Perhaps both regions make up the true domain. Region A may have a general disruptive effect on the starch granule structure, as suggested by Hayashida et al. [7]. Region B may specifically bind in a preferred orientation to the granular substrate, leading to a tightly bound complex between the protein and the starch granule and thus decreasing the activation energy for catalysis. This would explain the promotion of granular

starch hydrolysis by glucoamylase 1 with region A but the inhibition of digestion with G1C containing both regions A and B.

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