Production, purification and characterization of the catalytic domain of glucoamylase from *Aspergillus niger*

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The catalytic domain of glucoamylases G1 and G2 from *Aspergillus niger* is produced *in vitro* in high yield by limited proteolysis using either subtilisin Novo or subtilisin Carlsberg. Purification by affinity chromatography on an acarbose–Sepharose column followed by ion-exchange chromatography on HiLoad Q-Sepharose leads to separation of a number of structurally closely related forms of domain. The cleavage occurs primarily between Val-470 and Ala-471 as indicated by C-terminal sequencing, whereas the N-terminus is intact. Subtilisin Carlsberg, in addition, produces a type of domain which is hydrolysed before Ser-444, an O-glycosylated residue. This leaves the fragment Ser-444–Val-470 disulphide-bonded to the large N-terminal part of the catalytic domain. Subtilisin Novo, in contrast, tends to yield a minor fraction of forms extending approx. 30–40 amino-acid residues beyond Val-470. The thermostability is essentially the same for the single-chain catalytic domain and the original glucoamylases G1 and G2, whereas the catalytic domain cut between Ser-443 and Ser-444 is less thermostable. For both types of domain the kinetic parameters, K_m and $k_{cat.}$, for hydrolysis of maltose are very close to the values found for glucoamylases G1 and G2.

INTRODUCTION

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) catalyses the release of glucose from the non-reducing ends of starch and related poly- and oligo-saccharides [1]. Like most amylolytic enzymes, fungal glucoamylases are multidomain proteins; the organization of the individual domains along the polypeptide chain in glucoamylases depends on the species [2]. Short forms of different glucoamylases lacking either an N-terminal or a C-terminal domain are unable to adsorb on to and degrade starch granules [3–6].

The glucoamylase G1 from *Aspergillus niger* consists of three parts: (1) Ala-1–Thr-440, that contains the catalytic site; (2) Ser-441–Thr-511, a highly O-glycosylated linker segment, and (3) Pro-512–Arg-616, a C-terminal domain responsible for raw starch binding and dispensable for activity [4,7]. The starch-binding domain was recently isolated in low yield following release by proteolysis *in vitro* [8]. Limited proteolysis has previously led to formation of catalytically active forms of glucoamylases from related species, but their covalent structure remains to be determined [9,10]. For *A. niger* glucoamylase a combination of chemical modification and site-directed mutagenesis has provided much basic knowledge concerning essential residues involved in substrate binding and catalysis [11–14]. To improve our insight into the catalytic process a three-dimensional structure of the *A. niger* glucoamylase is required. Due to the architecture of the enzyme, the catalytic domain is the preferred species for crystallization experiments. Ultimately the studies on the separate domains will help in elucidating the significance of domain–domain interactions. Here we report the large-scale preparation of the catalytic domain from *A. niger* glucoamylases G1 and G2 using subtilisins and some chemical, stability and enzymic properties of the purified domains.

EXPERIMENTAL

Materials

A. niger glucoamylases G1 and G2 were prepared from AMG 200L (Novo Nordisk) [4]. Subtilisins types Novo and Carlsberg (EC 3.4.21.14) (Novo Nordisk), clostripain (EC 3.4.22.8) and chymotrypsin (EC 3.4.21.1) (Merck), thermolysin (EC 3.4.24.4) and trypsin (EC 3.4.21.4) (Sigma), and *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) (Miles) were all used without further purification. Papain (EC 3.4.22.2) was a laboratory preparation and carboxypeptidase Y (EC 3.4.16.1) was a gift from Carlbiotech. The acarbose–Sepharose was prepared as described earlier [11]. Chemicals used to prepare Tricine/SDS/polyacrylamide gels were electrophoresis purity reagents from Bio-Rad. The glucose oxidase kit was from Sigma. The glucose dehydrogenase kit and maltose monohydrate were from Merck. 4-Nitrophenyl α-D-glucopyranoside (pNPG) was from Boehringer–Mannheim. Water was drawn from a Milli-Q system (Millipore). If not otherwise stated chemicals were of analytical grade.

Preparation of the catalytic domain

In preliminary experiments either glucoamylase G1 or G2 (2 mg/ml) was incubated with chymotrypsin, trypsin, subtilisin, *S. aureus* V8 proteinase, clostripain, or thermolysin (0.2 mg/ml) in 10 mM ammonium bicarbonate/1 mM $CaCl_2$, pH 7.8, for 1 h or 6 h at 25 °C. Treatment with papain (0.2 mg/ml) was performed essentially as described above in 10 mM ammonium acetate, pH 5.0. To stop proteolysis aliquots of the reaction mixtures were heated at 100 °C for 5 min after adding the sample buffer and degradation of glucoamylase was followed by

Abbreviations used: glucoamylases G1 and G2, glucoamylase G1 (residues 1–616) and G2 (residues 1–512); SN, subtilisin Novo; SC, subtilisin Carlsberg; glucoamylases G1 SN or G2 SN and glucoamylases G1 SC or G2 SC, the single-polypeptide-chain forms of the catalytic domain from glucoamylase G1 or G2 produced by subtilisin Novo and subtilisin Carlsberg respectively; glucoamylases G1 SN′ or G2 SN′ and glucoamylases G1 SC′ or G2 SC′, the corresponding two-polypeptide-chain forms; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; pNPG, 4-nitrophenyl α-D-glucopyranoside.

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SDS/PAGE in 10–15% (w/v) gradient Phastgels (Pharmacia) which were run and stained according to the manufacturer's recommendations. For the large-scale preparation of the catalytic domain, glucoamylases G1 or G2 (2 mg/ml; 100 ml) were treated with either subtilisin Novo (SN) or subtilisin Carlsberg (SC) (0.4 mg/ml) as described above for 10 h. After incubation, subtilisin was inactivated by phenylmethanesulphonyl fluoride (PMSF) added to a final concentration of 1 mM. The progress of the proteolysis of glucoamylase was monitored in PMSF-treated aliquots by activity measurement using maltose as substrate [4] and by Tricine/SDS/PAGE.

Purification of the catalytic domain

Enzymically active glucoamylase was isolated from the PMSF-treated reaction mixture by affinity chromatography on an acarbose–Sepharose column [11]. The eluted mixture of glucoamylase forms was dialysed against 20 mM piperazine/0.002% (w/v) NaN_3, pH 5.3, at 5 °C and further fractionated at room temperature by ion-exchange chromatography using a HiLoad Q-Sepharose 26/10 f.p.l.c. column (Pharmacia). The column was pre-equilibrated in 20 mM piperazine/0.15 M NaCl/0.002% (w/v) NaN_3, pH 5.3, before sample application. The different molecular forms of glucoamylase were eluted with a NaCl gradient made from 20 mM piperazine/0.002% (w/v) NaN_3, pH 5.3, and 20 mM piperazine/0.6 M NaCl/0.02% (w/v) NaN_3, pH 5.3, respectively, using two P-500 pumps (Pharmacia). The pumps were programmed to give the following NaCl concentrations in linear segments: initial time (0.15 M NaCl); 65 min (0.39 M NaCl); 66 min (0.6 M NaCl); 85 min (0.6 M NaCl); 86 min (0.15 M NaCl); and 110 min (0.15 M NaCl). The elution was performed at a flow rate of 2.2 ml/min and monitored by u.v. absorbance at 276 nm. Fractions (4.4 ml) were collected and aliquots were removed for assay of activity toward pNPG and for Tricine/SDS/PAGE respectively.

Analytical techniques

Protein concentration was determined spectrophotometrically at 280 nm using $\epsilon = 1.37 \times 10^5\ M^{-1} \cdot cm^{-1}$ for glucoamylase G1 and $\epsilon = 1.09 \times 10^5\ M^{-1} \cdot cm^{-1}$ for glucoamylase G2 and all domain types [15].

The glucoamylase G1 SN domain (20 mg/ml) was reduced, alkylated by 2-vinylpyridine and cleaved by CNBr [7,16]. The fragments obtained were separated on a column of Bio-Gel P-30 (0.9 cm × 38 cm) by elution with 10% (v/v) acetic acid at a flow rate of 5.9 ml/h.

Protein hydrolysates were made as described [17] and the amino-acid contents were quantified by ninhydrin detection using a Pharmacia LKB-Alpha Plus amino-acid analyser according to the manufacturer's instructions. N-terminal sequencing was performed either on an Applied Biosystems protein sequencer model 470A or model 477A, both equipped with an on-line Applied Biosystems PTH analyser model 120A, using standard programs supplied by the manufacturer [18].

The glucoamylase G1 SN and G1 SC′ domains (5–7 mg/ml) were subjected to C-terminal-sequence analysis using carboxypeptidase Y (0.6 mg/ml) at room temperature in 50 mM Mes, pH 6.3/0.5% (w/v) SDS/100 μM norleucine. Aliquots were removed at appropriate time intervals and acidified to pH 1 with 1 M HCl. The released amino-acid residues were determined by amino-acid analysis and quantified based on norleucine recovery and the concentration of the protein substrate determined by amino-acid analysis of aliquots removed before addition of carboxypeptidase Y.

Tricine/SDS/PAGE of fractions from ion-exchange chromatography was performed in 0.75 mm gels consisting of a separation gel (5 cm × 16 cm), a spacer gel (6 cm × 16 cm), and a stacking gel (3 cm × 16 cm) containing 16.5% (w/v), 8.3% (w/v), and 4% (w/v) acrylamide respectively, all containing 1.5% (w/v) bisacrylamide. The gels were cast and electrophoresed at 13 °C using a Bio-Rad Protean II xi cell and power supply model 1000/500 according to [19,20]. Protein was denatured before application to the gel by boiling in sample buffer with or without dithiothreitol (DTT) and subsequently alkylated with iodoacetamide [19–21]. The gels were stained with Coomassie Brilliant Blue R 250 and destained as described [19].

Enzyme assays

Activity measurement, using maltose as substrate, was performed as described previously [4], and the quantification of liberated glucose was carried out by using a glucose oxidase kit [22]. The PC program Enzfitter [23] was used to calculate Michaelis–Menten parameters, K_m and $k_{cat.}$, from initial rates of the glucoamylase (1.8 nM)-catalysed hydrolysis of maltose (0.2–10 mM) in 50 mM sodium acetate (pH 4.4) at 45 °C. The glucoamylase concentration was determined by amino-acid analysis. The assay of fractions from the ion-exchange chromatography using pNPG as substrate was performed in microtitre plates and monitored by absorbance measurement at 405 nm essentially as described [24].

Thermostability

The thermostability of active glucoamylase domain (0.9 μM) at pH 4.3 was assessed by incubation at various temperatures for 5 min followed by activity measurements using maltose as substrate [4,25].

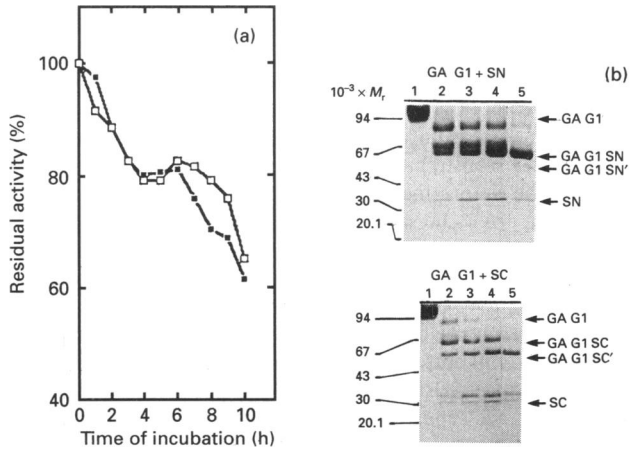

Figure 1 Limited proteolyses of glucoamylase G1 from *A. niger* by SN and SC, followed by activity measurement and Tricine/SDS/PAGE

(**a**) The residual activity of glucoamylase (GA) G1 treated with SN (□) or SC (■) for 0–10 h of incubation, was measured using maltose (15 mM) as substrate at pH 4.3 and 25 °C. The activity at 0 h is set to 100%. (**b**) Tricine/SDS/PAGE analysis of reduced and alkylated samples from incubation mixtures of glucoamylase G1 with SN or SC, after 0, 1, 2, 3, and 10 h (lanes 1–5). Each lane is loaded with the equivalent of 50 μg of uncleaved glucoamylase G1. Positions of different forms of glucoamylases (GA G1, GA G1 SN, GA G1 SN′, GA G1 SC, GA G1 SC′), subtilisins (SN, SC), and M_r-standards are indicated by arrows and lines respectively.

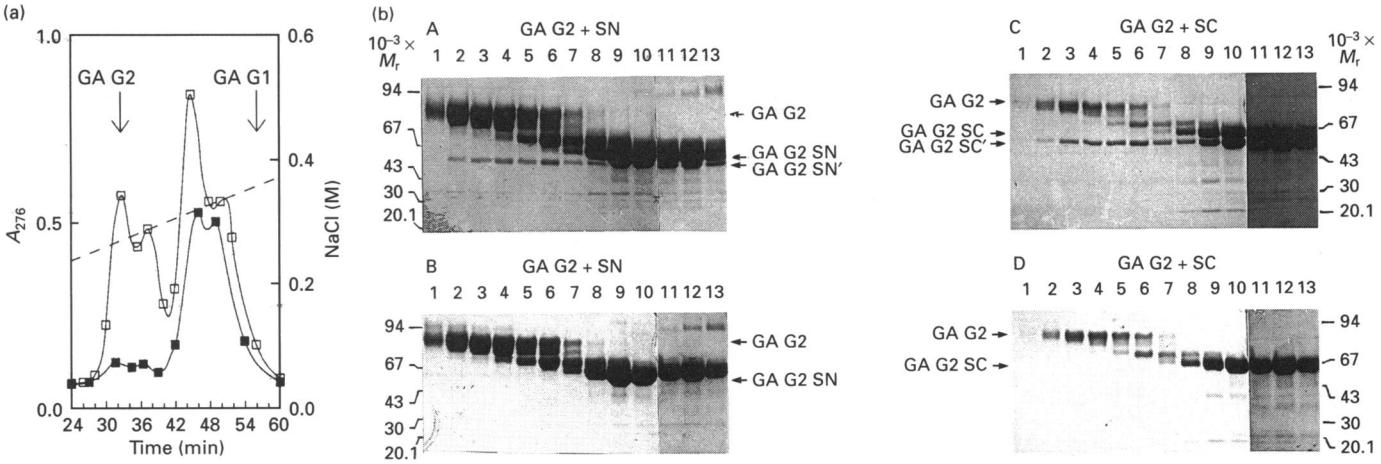

Figure 2 Elution profiles and Tricine/SDS/PAGE analysis of fractions from HiLoad Q-Sepharose chromatography of glucoamylase G2 from *A. niger* after cleavage with SN and SC

(**a**) Ion-exchange chromatography on HiLoad Q-Sepharose of acarbose–Sepharose-purified glucoamylase (GA) G2 after cleavage with either SN (□) or SC (■) for 10 h. The NaCl gradient is indicated (---) (for conditions see the Experimental section). The elution positions corresponding to uncleaved glucoamylases (GA G2, 33 min and GA G1, 57 min) are shown by arrows. (**b**) Tricine/SDS/PAGE analysis of fractions between 30 and 54 min (lanes 1–13) from the HiLoad Q-Sepharose chromatography of glucoamylase G2 cleaved by either SN (panels A and B) or SC (panels C and D) were done under reducing (panels A and C) and non-reducing (panels B and D) conditions, using M_r standards as reference. The lanes were loaded with the equivalent of 6–72 μg and 1–41 μg of uncleaved glucoamylase G2 for the experiments with SN and SC respectively. The positions of different forms of glucoamylases (GA G2, GA G2 SN, GA G2 SN′, GA G2 SC, and GA G2 SC′) and M_r standards are indicated by arrows and lines respectively.

RESULTS

Limited proteolysis of glucoamylase

Enzymically active domain forms glucoamylases G1 SN and G1 SC′ are produced in high amounts by 10 h of incubation of native glucoamylases G1 or G2 (results not shown) from *A. niger* with SN and SC respectively [Figures 1(a) and 1(b)]. About 50% of the initial glucoamylase protein and activity was recovered after affinity chromatography on an acarbose–Sepharose column. This loss of approx. 50% of the initial activity (Figure 1a) was the result of further degradation, since the different kinds of purified catalytic domain retained activity similar to uncleaved glucoamylases G1 and G2. Typical recoveries after ion-exchange chromatography were 16% and 23% for domains obtained by SC and SN respectively. Papain was much less efficient than either of the subtilisins in producing glucoamylase catalytic domain, while the other proteinases tested did not cause any significant conversion, as judged from SDS/PAGE analysis (results not shown).

Chemical properties of the catalytic domain

Glucoamylases G1 and G2 migrate during Tricine/SDS/PAGE with $M_{r,app.}$ values of 94000 and 85000 respectively. Based upon the amino-acid sequence the protein parts of glucoamylases G1 and G2 have M_r values of 65793 and 54333 respectively. The carbohydrate content of both enzymes amounts to 16126, resulting in M_r values for glucoamylases G1 and G2 of 81919 and 70459 respectively [7].

Analysis of the proteolysis products of glucoamylase G1 (results not shown) and glucoamylase G2 after separation on a HiLoad Q-Sepharose column (Figure 2a) was done by Tricine/SDS/PAGE under non-reducing conditions and indicates that SN and SC yield domains having essentially the same $M_{r,app.}$ of 63000–65000 [Figure 2(b), panels B and D, lanes 9–13]. In addition, distinct domain forms are produced having estimated $M_{r,app.}$ values of 70000 and 76000, i.e. values intermediate to those of glucoamylase G2 and the dominant domain. This is most pronounced in the preparation made with SN [compare Figure 2(b), panels B and D]. Both SN and SC produced domains which behave as a single polypeptide chain under non-reducing conditions [Figure 2(b), panels B and D]. However, when the proteins are analysed under reducing conditions [Figure 2(b), panels A and C; Figure 1(b)] an additional band of approx. $M_{r,app.}$ 51000–55000 appears in the gels [glucoamylases G2 SN′ and G2 SC′ in Figure 2(b), panels A and C], indicating that a fraction of the catalytic domain of $M_{r,app.}$ 63000–65000 is composed of two polypeptide chains which are linked together through a disulphide bond.

A population of catalytic domains, comprising two dominant types, thus results from the proteolysis of glucoamylases G1 and G2 by SN or SC. One of these of these has the 63000–65000-$M_{r,app.}$ polypeptide chain intact. The other major product, which is mainly produced by SC [Figure 2(b), A and C], has a cut in the polypeptide chain and is represented in Tricine/SDS/PAGE by bands of $M_{r,app.}$ 63000–65000 (non-reducing conditions) and $M_{r,app.}$ 51000–55000 (reducing conditions). The fragmentation is consistent with glucoamylase G1 SN and glucoamylase G1 SC′ having almost identical amino-acid compositions (Table 1). Furthermore, it is possible to have cleavage of glucoamylase molecules at this extra site before the attack producing the C-terminus of the dominant forms of the catalytic domain [Figure 2(b), panels A, C and B, D, lanes 2–4]. Only part of the glucoamylase molecules, however, seems to be susceptible to hydrolysis at the extra cleavage site, as judged from the lower N-terminal yields found for the sequence starting at Ser-444 and from the molecular species revealed by SDS/PAGE of reduced and alkylated samples. Intact glucoamylase G1, present due to a trace contamination from the previous acarbose–Sepharose chromatography purification, migrates with an $M_{r,app.}$ of 94000, as seen in lanes 11–13 on Figure 2(b), panels A and B. It had no influence on the further experiments.

The N-terminal sequence of a glucoamylase G1 SN preparation

Table 1 Amino-acid composition of catalytic domain resulting from cleavage of glucoamylase G1 from *A. niger* with SN or SC

The enzymes were hydrolysed and analysed as described in the Experimental section. Values for threonine, serine, valine and isoleucine have not been corrected for destruction or incomplete hydrolysis. Abbreviations: GA, glucoamylase; n.d., not determined.

	Molecular form (residues/mol)		
Amino acid	GA G1 SN	GA G1 SC′	GA G1 (1–470)*
Aspartic acid	56.4	57.9	35
Asparagine	–	–	22
Threonine	37.2	35.2	39
Serine	55.7	52.9	63
Glutamic acid	32.3	31.3	18
Glutamine	–	–	15
Proline	15.9	15.4	16
Glycine	36.2	35.0	35
Alanine	51.0	49.4	51
Valine	28.4	29.2	31
Methionine	1.7	1.6	2
Isoleucine	15.3	16.5	18
Leucine	36.8	36.4	37
Tyrosine	20.8	21.2	21
Phenylalanine	18.7	18.9	18
Histidine	4.2	4.3	4
Lysine	8.1	7.9	8
Arginine	14.8	15.1	15
Cysteine	n.d.	n.d.	7
Tryptophan	n.d.	n.d.	15
Total	433.5	428.2	470

* The composition of glucoamylase from Ala-1 to Val-470 deduced from the sequence [7].

Table 2 Amino-acid residues released by carboxypeptidase Y from the catalytic domain made from *A. niger* glucoamylase G1 by either SN or SC

See the Experimental section for incubation conditions and calculations. Abbreviation: GA, glucoamylase.

		Molecular form (residues/mol)	
Incubation (min)	Amino acid	GA G1 SN	GA G1 SC′
5	Valine	0.4	1.0
	Isoleucine	0.1	0.3
	Serine	0.2	0.7
	Alanine	0.1	0.6
80	Valine	0.7	1.3
	Isoleucine	0.2	0.8
	Serine	0.6	1.1
	Alanine	0.5	0.9

was found, over five cycles, to be identical with that of glucoamylase G1, whereas glucoamylase G1 SC′ over 20 cycles, in addition to the glucoamylase G1 N-terminal sequence, revealed, in high proportion, the sequence starting by the glycosylated Ser-444. The C-terminal CNBr-cleaved peptide was prepared from glucoamylase G1 SN (see the Experimental section) and shows an amino-acid composition compatible with a C-terminus of that domain form at Val-470 (results not shown). C-terminal sequencing using carboxypeptidase Y was done on a preparation of glucoamylase G1 SN, that contained very small amounts of the glucoamylase G1 SN′ form, and a preparation of glucoamylase G1 SC′, rich in the two-polypeptide-chain form, respectively (Table 2). Valine is released first, followed by serine, alanine and isoleucine. This pattern confirms that Val-470 is the C-terminal residue in the two major forms of catalytic domain, glucoamylase G1 SN and glucoamylase G1 SC′. Extending the incubation with carboxypeptidase Y from 5 min to 80 min resulted in additional release of these residue types, in support of cleavages occurring before both Ser-444 and Ala-471. The domain preparation made with SC seems to be more readily attacked by carboxypeptidase Y, while some additional cleavage seems to occur of glucoamylase G1 SN during the carboxypeptidase Y treatment.

The calculated M_r values for Ala-1–Val-470 and Ala-1–Ser-443, migrating with $M_{r,app.}$ of 63000–65000 and 51000–55000 (Figure 2b), are 58820 and 53276 respectively. This unusually low mobility, given by different forms of glucoamylase, seems to be caused by the glycosylated linker region.

Table 3 Kinetic parameters for hydrolysis of maltose at pH 4.4 and 45 °C by the catalytic domain resulting from cleavage of glucoamylase G1 from *A. niger* by either SN or SC

See the Experimental section for conditions. Abbreviation: GA, glucoamylase.

Molecular form	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
GA G1	9.0±0.10*	1.0±0.10	9.0
GA G1 SN	9.0±0.13*	1.1±0.065	8.2
GA G1 SC′	8.4±0.13*	1.2±0.075	7.0

* S.D., average of three experiments.

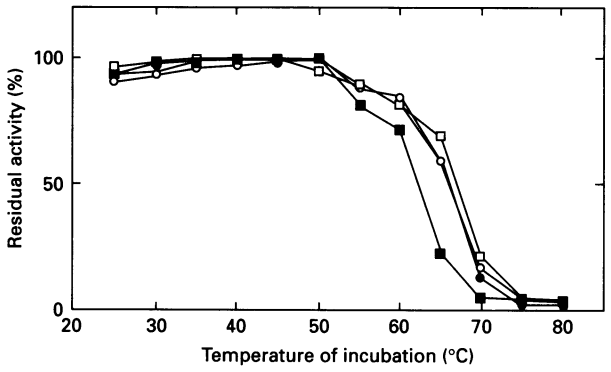

Figure 3 Thermostability of different domain forms and of glucoamylase G1 and G2 from *A. niger*

The residual activity of glucoamylase G1 (○), G2 (●), G1 SN (□), and G1 SC′ (■) have been measured with maltose as substrate at pH 4.3 and 25 °C after incubation of the enzyme at the temperatures indicated (see the Experimental section). The highest retained activity for each glucoamylase species is set to 100%.

Enzymic and thermostability properties of the catalytic domain

The kinetic parameters, K_m and $k_{cat.}$, for hydrolysis of maltose catalysed by glucoamylase G1 SN indicated a marginal reduction

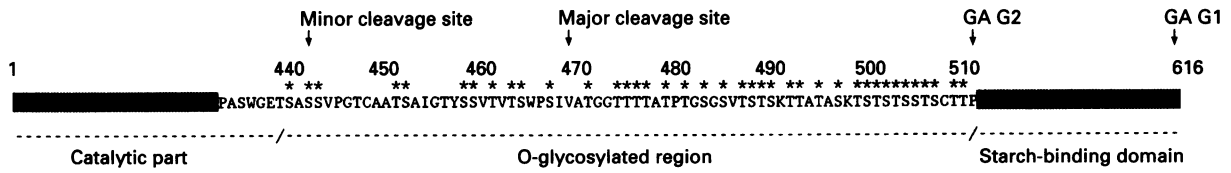

Figure 4 Schematic representation of the proteolysis of glucoamylases G1 and G2 from *A. niger* by SN and SC

The amino-acid sequence between residues Pro-434 and Pro-512 is shown (single letter code). The C-terminal residue(s) in a cleavage product is marked with arrow(s). O-glycosylated serine and threonine residues are indicated by * according to [7]. Cleavage of glucoamylase G1 or G2 only at the major cleavage site by SN or SC produces glucoamylase G1 SN, G2 SN, G1 SC, or G2 SC respectively, whereas cleavage at both minor and major cleavage sites by SN or SC produces glucoamylase G1 SN′, G2 SN′, G1 SC′, or G2 SC′ respectively (see text).

in the activity, whereas a small but significant reduction was found for glycoamylase G1 SC′ compared with glucoamylase G1 (Table 3). The single-polypeptide-chain form of the domain, glucoamylase G1 SN, also retained the thermostability, $T_{m,app.}$ being 67 °C, whereas glucoamylase G1 SC′ was considerably less stable, having a $T_{m,app.}$ value of 62 °C (Figure 3).

DISCUSSION

A dominant form of a catalytically active domain of *A. niger* glucoamylase arises by the limited proteolysis by SN and SC of glucoamylases G1 or G2 at the peptide bond between Val-470 and Ala-471. SN and SC have the capacity to cleave before Ser-444 also. This is by far most pronounced with SC and probably requires a non-glycosylated Ser-443. Both of these two forms of the catalytic domain are produced by cleavage in an unusual Ser/Thr-rich linker region (Ser-441–Thr-511) where about half of the amino-acid side-chains are O-glycosylated; the frequency of such residues increases sharply from Thr-473 (Figure 4) [7]. The second cleavage site is located before the O-glycosylated serine Ser-444, at the presumed border between the catalytic part and the Ser/Thr-rich region. In the three-dimensional structure of a closely related glucoamylase the corresponding bond is found in a turn [26]. The amino-acid composition of the glucoamylase G1 SC′ preparation and the rapid release of serine and alanine by carboxypeptidase Y both suggest that the residues from Trp-437 to Ser-443 may have been lost due to further subtilisin proteolysis to yield a second C-terminus at Ser-436, rather than at Ser-443.

The results of the present preparation of the catalytic domain excellently complement those of the isolated starch-binding domain, Ala-471–Arg-616 [8]. Our yield of the catalytic domain is 8–12-fold higher, however, than the recovery of Ala-471–Arg-616, the raw starch-binding domain. We speculate that the forms intermediate in size to intact glucoamylase and the major catalytic-domain forms may have C-termini at positions 498 and 508 respectively, thus matching the C-terminal peptides 499–616 and 509–616, reported to appear in addition to the dominant fragment 471–616 [27]. The three-dimensional structure of a form of *Aspergillus awamori* var. X100 glucoamylase has recently been determined [26]. This enzyme, which is homologous to *A. niger* glucoamylase residues 1–470, is present in high amounts in the culture medium [28]. However, the corresponding glucoamylase form is not found in the crude extract from *A. niger* used for the present preparation of the catalytic domain. A catalytic-domain preparation of *A. niger* glucoamylase G1 SN, containing 5–10% of the two-polypeptide-chain form glucoamylase G1 SN′, has recently been crystallized (B. Stoffer unpublished work). Since several active-site mutants are available of *A. niger* glucoamylase [13,14] we plan to do preparative-scale production of the corresponding domains for crystallization and structural analysis by X-ray diffraction.

Previously genetic truncation studies on *A. niger* glucoamylase resulted in an active and stable form with a C-terminus at Thr-482 [29], while a form with its C-terminus at Ser-460 was highly unstable, indicating that the structural integrity of the active site in domains cut between Ser-443 and Ser-444 probably depends on the retained C-terminal peptide, Ser-444–Val-470, which is disulphide-linked to helix H7 in the catalytic domain [26]. Because limited proteolysis of both glucoamylase G1 and G2 from *A. niger* results in the same catalytically active domain, the C-terminal starch-binding domain, Thr-513–Arg-616, present only in glucoamylase G1 [7,16], seems not to be in intimate contact with the catalytic domain. This conclusion is consistent with n.m.r. studies that suggest a model in which the two domains are linked by a semi-flexible rod and have a significant amount of independent motion [27]. Since domain shuffling has occurred in fungal glucoamylases [5,7,30,31], and the C-terminal residues in the cases of *Saccharomyces cerevisiae*, *Rhizopus oryzae*, and *Saccharomycopsis fibuligera* glucoamylases aligned with *A. niger* glucoamylase residues Lys-404, Ala-435, and Ser-436 respectively [30], the question arises whether the folding pattern of the different glucoamylase catalytic domains in its entirety closely follows that of the *A. niger* glucoamylase.

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