

Characterization of a glucoamylase G2 from *Aspergillus niger*

Birte SVENSSON¹, Kjeld LARSEN¹ and Alf GUNNARSSON²

¹ Department of Chemistry, Carlsberg Laboratory, Copenhagen

² Department of Carbohydrate Chemistry, Chemical Center, University of Lund

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Peptide fragments were generated by enzymic or chemical degradation of the small form, G2, and the large form, G1, of *Aspergillus niger* glucoamylase (EC 3.1.2.3). The G2 form was either identical to residues Ala¹–Pro⁵¹² or to Ala¹–Ala⁵¹⁴ of the G1 polypeptide chain containing 616 amino acid residues. Structural analysis of the *O*-linked carbohydrates from the 70-amino-acid-residues long extensively glycosylated segment of G2 revealed no significant differences in the contents of single mannose and oligosaccharide units in comparison to the corresponding region of G1. The results suggest that the present G2 form has been generated by limited proteolysis of the larger G1. In contradistinction to this, a recently reported splicing out of an intervening sequence from G1 mRNA leads to a smaller mRNA coding for a G2 protein product with a different COOH-terminal sequence than the G2 form described in the present work [Boel et al. (1984) *EMBO J.* 3, 1097–1102].

Mold glucosamylases (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) are of industrial importance in the production of sugar from starch [1]. They often exist in multiple forms [2–4]. The primary structure of the large form, glucoamylase G1, from *Aspergillus niger* has been determined recently [5], and the nucleotide sequences of the cloned cDNA and gene fully agree with the protein studies [6, 7]. As is generally the case with fungal glucoamylases [3], only the larger form, G1, of *A. niger* glucoamylase possesses the capacity to adsorb onto and to digest raw starch, while both G1 and the smaller G2 are essentially equally active towards soluble polysaccharide and oligosaccharide substrates [3, 8]. It was previously suggested that the smaller form G2, in comparison to G1, lacks a COOH-terminal segment of an apparent M_r 10000 [6, 8]. In the present study glucoamylase G2 isolated from a commercial preparation of the enzyme has been characterized with respect to amino acid sequence and structure of *O*-linked carbohydrate units. The results confirm that the glucoamylase G2 investigated consists of two molecular species identical to the residues 1–512 and 1–514 of glucoamylase G1 which has 616 residues.

EXPERIMENTAL PROCEDURES

Materials

Glucoamylase G1 and G2 from *Aspergillus niger* were isolated as previously described [8] from AMG 200 L (batch ASN 5192, Novo Industries, Bagsvaerd, Denmark). Cellex P was from Bio-Rad (Richmond, CA) and α -chymotrypsin from

Sigma (St Louis, MO). Trifluoroacetic acid and trifluoroacetic acid anhydride were from Janssen Chimica (Beerse, Belgium). Endopeptidase-free carboxypeptidase Y was prepared in this laboratory. Other products and chemicals were obtained as earlier published [5, 9, 10].

Preparation and isolation of peptide fragments

G1 and G2 were reduced and 2-pyridylethylated as earlier reported [9, 11] to give 2-pe-G1 and 2-pe-G2. At the end of the reaction 5% (v/v) 2-mercaptoethanol was added to destroy excess 2-vinylpyridine. The reaction mixtures were then dialyzed against 0.1% 2-mercaptoethanol in 10% ethanol followed by water and stored at -18°C .

Aliquots of the 2-pe-G1 and 2-pe-G2 preparations were lyophilized, subjected to cyanogen bromide cleavage and the fragments separated and rechromatographed on DEAE-Sephacel as earlier described [5]. Non-adsorbed material, only found from 2-pe-G2, was further purified on Cellex P.

Prior to digestion by *S. aureus* V8 protease, aliquots of the stock suspensions of 2-pe-G1 and 2-pe-G2 were diluted to $5\text{ mg} \cdot \text{ml}^{-1}$ in 0.1 M sodium phosphate pH 7.6 and then incubated with the enzyme ($0.05\text{ mg} \cdot \text{ml}^{-1}$) for 20 h at 40°C . The digests were subsequently diluted with 9 vol. 0.050 M Tris/HCl, 0.5 M NaCl pH 7.4 and the glycopeptides adsorbed onto ConA-Sepharose and then eluted and desalted as previously reported [9]. Portions (10 mg) of glycopeptides were subjected to gel filtration on Bio-Gel P-60 ($1.5 \times 90\text{ cm}$) in 0.2 M ammonium bicarbonate pH 7.8.

The tryptic digestion of 2-pe-G2 was performed as published for 2-pe-G1 and the highly glycosylated, long tryptic fragment (T2-1) was isolated with the aid of ConA-Sepharose while other tryptic fragments were purified by a combination of Bio-Gel P-100 chromatography and RP-HPLC essentially as before [9, 12]. Chymotryptic sub-fragments were prepared from a mixture of two tryptic fragments ($3\text{ mg} \cdot \text{ml}^{-1}$), performing the digestion at a substrate/enzyme mass ratio of 30 in 0.1 M ammonium bicarbonate pH 7.8 for 5 h at 37°C , and subsequently purified by RP-HPLC on a J. T. Baker Wide-Pore C₁₈ column

Correspondence to B. Svensson, Kemisk Afdeling, Carlsberg Laboratorium, Gamle Carlsberg Vej 10, DK-2500 København, Denmark

Abbreviations. G1 and G2 designate the large and the small forms of glucoamylase from *A. niger* [8]; 2-pe, 2-pyridylethyl; RP-HPLC, reversed-phase high-performance liquid chromatograph; GLC, gas-liquid chromatography; MS, mass spectrometry.

Enzymes. Glucoamylase (EC 3.2.1.3); trypsin (EC 3.4.21.4); chymotrypsin (EC 3.4.21.1); *Staphylococcus aureus* V8 protease (EC 3.4.21.19).

(4.6 × 250 mm) in the trifluoroacetic acid/1-propanol solvent system [9, 12]. RP-HPLC rechromatography was occasionally performed with a Waters Nova-Pak Radial-Pak C₁₈ 5-μm column (8 × 100 mm) eluted isocratically with 0.1% trifluoroacetic acid in 9% (v/v) CH₃CN for 12 min followed by a linear gradient up to 63% (v/v) CH₃CN over 60 min at a flow rate of 1.5 ml min⁻¹. The effluent was monitored at 220 nm.

COOH-terminal determination

The fragment generated by *S. aureus* V8 protease, E1, comprising the COOH-terminus of G2, and the corresponding fragment from G1 were deglycosylated for 3 h at 0°C with anhydrous hydrogen fluoride in the presence of anisole and purified by gel filtration on Bio-Gel P-6 in 30% acetic acid as described [9, 13]. After redissolution in 0.05 M Mes pH 4.5, carboxypeptidase Y was added to give an enzyme/substrate molar ratio of 1:1000. The digestions were stopped after 60 min by acidification to pH 2.0 using acetic acid and the released amino acids were determined utilizing a Durrum D-500 amino acid analyzer.

Analytical procedures

Methods for determination of amino acid compositions, contents of *O*-glycosylated amino acid residues and neutral carbohydrates have been reported earlier [9, 14–16]. NH₂-terminal sequencing was performed with a Beckman 890 C sequencer [17, 18]. Monosaccharides were analysed by GLC as alditol acetates after hydrolysis of the glycopeptide with 4 M trifluoroacetic acid for 4 h at 100°C as earlier described [10, 19, 20]. Oligosaccharides released by trifluoroacetolysis from 3–5 mg of E1 fragments were analysed as permethylated oligosaccharide alditols by GLC-MS using a VG Mass Lab 12/250 quadrapole instrument [10].

Peptide nomenclature

The CB1–CB3, E1 and T2-1 from G1 and G2 are named as previously reported for G1 [5, 9]. Other tryptic fragments and chymotryptic subfragments from G2 are referred to by the pool number from Bio-Gel P-100 elution [9] followed by an RP-HPLC peak number. C signifies chymotryptic subfragments.

RESULTS

Cyanogen bromide fragments

The elution patterns for the cyanogen bromide fragments from G1 and G2 in anion-exchange chromatography on DEAE-Sephacel are compared in Fig. 1A and 1B. The NH₂-terminal, CB1, and the central, CB2, cyanogen bromide fragments behaved very similarly and no significant differences were detected between the G1 and G2 fragments in their respective NH₂-terminal sequences, amino acid and carbohydrate contents (data not shown). In contrast, however, the COOH-terminal fragment CB3-G2 was much less acidic than CB3-G1 (compare Fig. 1A and 1B). Consequently the material in pool CB3-G2 (Fig. 1B), was poorly retained on DEAE-Sephacel, while cation-exchange chromatography on Cellex P (Fig. 2) resolved it into three peaks, CB3-G2 (1–3), which all had essentially identical amino acid composition (data not shown). Presumably the different elution

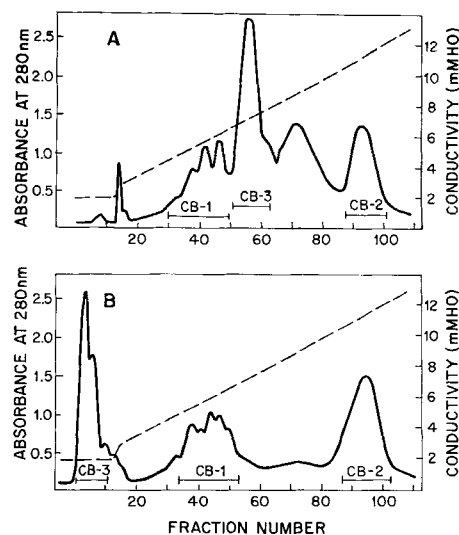


Fig. 1. Separation of cyanogen bromide fragments of glucoamylases (A) G1 (260 mg) and (B) G2 (200 mg) on DEAE-Sephacel. The column (1.6 × 70 cm) was equilibrated in 7 M urea, 0.05 M Tris/HCl pH 8.0 and eluted with a linear gradient of 0–0.2 M guanidinium chloride in the same buffer (2 × 600 ml) at a flow rate of 40 ml · h⁻¹. Fractions of 11 ml were collected and the absorbance at 280 nm (—) and the conductivity (---) were measured. The pools indicated by horizontal bars were rechromatographed in the same system and the fragments desalted prior to chemical characterization.

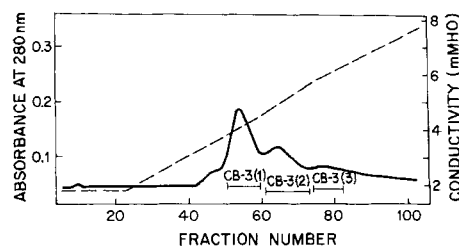


Fig. 2. Separation of fragments in pool CB3 from glucoamylase G2. Pool CB3 (see Fig. 1B) was separated on a column of Cellex P (1.6 × 20 cm) equilibrated in 7 M urea, 0.025 M phosphoric acid pH 3.1 and eluted with 50 ml of this buffer followed by a linear gradient of 0–0.3 M potassium chloride (2 × 200 ml) at a flow rate of 30 ml · h⁻¹. Fractions of 2.6 ml were collected and the absorbance at 280 nm (—) and the conductivity (---) were measured. The pools indicated by horizontal bars were desalted prior to amino acid analysis. The chemical composition of CB3-G2 (1) is shown in Table 1.

positions of CB3-G2 (1–3) reflect variations in contents of amino acid amides or in other post-translationally modified positions rather than differences in the translated amino acid sequence. The NH₂-terminal sequences of CB3-G1 and CB3-G2 (1) were identical while differences were observed between the amino acid compositions. Thus CB3-G2 (1) contained less aspartic acid/asparagine, glutamic acid/glutamine, isoleucine, leucine and tyrosine and no phenylalanine (Table 1). This composition agrees with G2 consisting of residues Ala¹–Pro⁵¹² and thus terminating right after the long region with the high frequency of *O*-glycosylated positions (Fig. 2).

Free amino acids were not liberated from G2 by carboxypeptidase Y probably due to carbohydrate substituents near the COOH-terminal residue. Both deglycosylated G2 and CB3-G2 are very poorly soluble in aqueous buffers while two smaller deglycosylated polypeptide

Table 1. Composition of COOH-terminal fragments from glucoamylase G2 and the corresponding G1 fragments

The values in parenthesis were obtained from the G1 sequence [5, 6]. Values for threonine and serine were extrapolated to zero time of hydrolysis. Values for valine and isoleucine were those after 72 h of hydrolysis. 2-Pe-cysteine = 2 Pyridylethylcysteine [11]. Tryptophan was determined from the sequence [5] or by 2nd derivative spectroscopy [16]. *O*-Glycosylated threonine and serine were determined by reductive β -elimination [14]. Neutral sugar was determined by the phenol/sulfuric acid procedure [15] using mannose as standard. n.d. = not determined

Amino acid	Residues/fragment								
	CB3 S399-P512/R616		CB3 difference (G1-G2)	E1 T440-P512/D520		E1 difference (G1-G2)	T2-1 N430-P512/519		T2-1 difference (G1-G2)
	G2	G1		G2	G1		G2	G1	
Aspartic acid	6.0	17.4 (17)	11	0.3	1.1 (1)	1	1.1	1.2 (1)	0
Threonine	25.4	41.3 (42)	16	24.1	26.2 (26)	2	23.4	25.8 (26)	2
Serine	23.2	34.6 (35)	11	17.4	17.2 (17)	0	19.4	19.6 (20)	0
Glutamic acid	4.8	15.0 (15)	10	0.2	0.0 (0)	0	1.3	0.9 (1)	0
Proline	4.5	8.9 (9)	4	3.8	3.7 (4)	0	4.5	4.8 (5)	0
Glycine	8.3	14.7 (14)	6	5.8	6.1 (6)	0	7.0	6.6 (7)	0
Alanine	13.0	21.0 (21)	8	8.2	9.9 (10)	2	8.6	10.9 (11)	2
Valine	7.0	14.6 (15)	8	4.9	6.6 (7)	2	6.2	8.5 (9)	2
Methionine	0	0 (0)	0	0	0 (0)	0	0	0 (0)	0
Isoleucine	2.0	7.1 (7)	5	2.0	2.0 (2)	0	1.9	1.9 (2)	0
Leucine	4.0	10.2 (10)	6	0.1	0.2 (0)	0	0.2	0.1 (0)	0
Tyrosine	2.7	9.0 (9)	6	1.2	1.3 (1)	0	0.9	1.1 (1)	0
Phenylalanine	0.0	3.9 (3)	4	0.0	1.3 (1)	1	0.1	1.0 (1)	1
Histidine	0	0 (0)	0	0	0 (0)	0	0	0 (0)	0
2-Pe-cysteine	1.4	2.5 (3)	1	1.3	1.4 (2)	0	1.4	1.4 (2)	0
Lysine	2.7	4.5 (5)	2	1.8	1.8 (2)	0	2.0	1.9 (2)	0
Arginine	2.8	5.5 (6)	3	0.0	0.1 (0)	0	0.0	0.0 (0)	0
Tryptophan	n.d.	7 (7)	0	1.9	2 (2)	0	1.8	2 (2)	0
Homoserine/homoserine lactone	0	0 (0)	0	0	0 (0)	0	0	0 (0)	0
<i>O</i> -Glycosylated threonine	23	21							
<i>O</i> -Glycosylated serine	17	16							
Neutral sugar	76	73							

fragments comprising the COOH-terminus of G2, namely T2-1 and E1 (described below), are fairly water-soluble. For this reason deglycosylated E1 was utilized for identification of the COOH-terminal sequence of G2 (see below).

Tryptic fragments and chymotryptic subfragments

The amino acid compositions of the long fragments T2-1 from G1 [9] and G2 (Fig. 3) which both carry a large number of *O*-glycosidically linked short carbohydrate units (Table 1) suggested Pro⁵¹² as the COOH-terminus of G2.

RP-HPLC fingerprints of peptide fragments were established verifying the rest of the G2 molecule. Tryptic fragments from 2-pe-G2 separated on Bio-Gel P-100 gave an elution profile similar to the 2-pe-G1 tryptic fragments (see Fig. 1 in [9]) and four pools (equivalent to pools 5, 6, 7, 8 from 2-pe-G1) containing medium-size and short peptides, were subsequently fractionated by RP-HPLC (Fig. 4a–d). Furthermore, the 2-pe-G2 fragments Asn¹⁶¹–Arg¹⁹⁴ and Ala¹⁹⁵–Arg²⁴¹ eluting first from Bio-Gel P-100 (equivalent to pool 1 from 2-pe-G1 [9]) were digested by chymotrypsin and the resulting subfragments were purified employing RP-HPLC (Fig. 4e). The amino acid compositions and NH₂-terminal sequences of the isolated tryptic and chymotryptic peptides (data not shown) matched the sequences listed in the legend of Fig. 4. Due to cross-contamination and co-elution several of the fragments were finally purified by rechromatography in the same or in a different system (described in Experimental Procedures) using more shallow, linear gradients of organic mobile-phase modifier (typically corresponding to an increase of 5–15% from the actual starting concentrations). Under

the conditions chosen for tryptic digestion, all of the fragments generated from 2-pe-G2 remained in solution and several so-called chymotryptic cleavages had occurred, as indicated in Fig. 4, of peptide bonds including the α -carboxyl groups of: Tyr⁵⁰, Tyr¹¹⁶, Trp¹²⁰, Trp¹⁴¹, Leu¹⁴², Tyr³¹⁷, Tyr³²⁹, Tyr³³³, Tyr³⁶³, Tyr³⁷⁰, Trp⁴¹⁷, Tyr⁴¹⁹, and Leu⁴²³. Peaks corresponding to Ser⁴¹⁸–Tyr⁴¹⁹ and Ala⁴²⁰–Leu⁴²³ have also been observed. It was previously noticed that a significant fraction of the G2 molecules has Asp⁴ at the NH₂-terminus [8], as reflected also in the fragment Asp⁴–Arg¹⁵ (peak 6-8). Furthermore some bonds were cleaved incompletely: Arg¹²²–Pro¹²³ (peaks 7-1, 7-7, 8-4) [5], Lys²⁷⁹–Glu²⁸⁰ (peak 6-5) [5] and Arg⁴²⁸–Arg⁴²⁹ (peak 8-2). With both the Wide-Pore and the Nova-Pak columns several examples were observed of fragments eluting at different retention times, thus Asp⁴¹⁴–Leu⁴²³ was purified from peaks 8-14 and 8-15, Tyr³⁰⁶–Trp³¹⁷ from 8-8, 8-10 and 8-11 and Ile²²³–Phe²²⁷ from C1-16, C1-17 and C1-18 (Fig. 4). Only the peptide fragments needed to account for the 429 NH₂-terminal residues of G2 are indicated in Fig. 3. Additional fragments are identified as shown for the appropriate peaks in the RP-HPLC chromatograms (Fig. 4).

S. aureus V8 protease fragments: identification of G2 COOH-terminus

The fractionations of glycopeptides obtained by *S. aureus* V8 digestion from both 2-pe-G1 and 2-pe-G2 on Bio-Gel P-60 resulted in the pure E1 fragments well separated from a few shorter glycopeptides (chromatograms not shown). Automated sequencing confirmed their NH₂-terminus to be

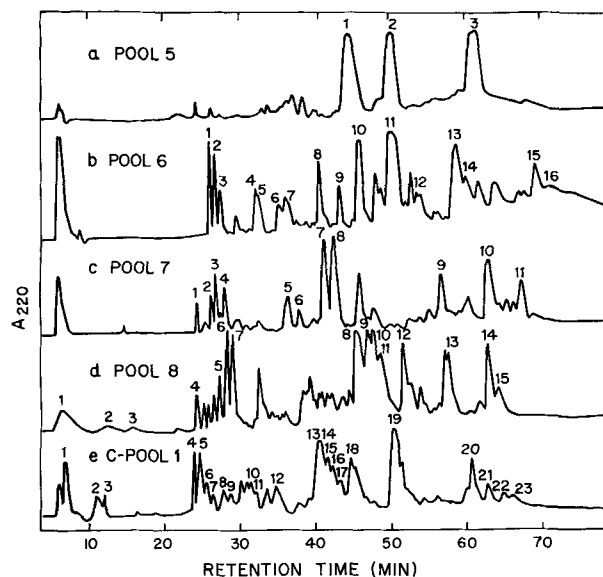


Fig. 4. RP-HPLC of tryptic peptides from 2-pe-G2. (a–d) Samples of peptide mixtures (0.5–1 mg) eluted in pools equivalent to the pools 5–8 from Bio-Gel P-100 (see [9], Fig. 1) were applied. The increasing pool numbers refer to increasing elution volumes. (e) The chromatogram C-POOL 1 shows the separation of chymotryptic subfragments of material eluting in the first peak from Bio-Gel P-100. For the RP-HPLC were used a Waters liquid chromatograph and a Wide Pore C₁₈ column from J. T. Baker Research Laboratories, described in a previous publication [9]. Elution was performed with a linear gradient over 80 min of 0–40% (v/v) 1-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml · min⁻¹. The effluent was monitored at 220 nm. Fractions were collected and lyophilized prior to amino acid analysis or rechromatography. Peptides in the indicated peaks were as follows. The pool numbers refer to the Bio-Gel P-100 elution. The peak numbers refer to the RP-HPLC chromatographies of the different pools eluted with different concentrations 1-propanol. Residues in parentheses have not been identified by sequencing, but were deduced from the published G1 sequence [5, 6] and the amino acid compositions of the present G2 fragments. Asterisks indicate glycosylated residues. The number of the COOH-terminal residue is given at the end in parentheses

Pool	Peak	1-Prop- anol eluate	Sequence of peptide	Pool	Peak	1-Prop- anol eluate	Sequence of peptide
		% (v/v)				% (v/v)	
5	1	21	T-F-A-D-G-F-V-S-I-V-E-T-H-A-A(S-N [*] -G-S-M-S-E-Q-Y-D-K) (404)	5	10		S(S-S-S-S-S-T-Y) (370)
	2	22	D-A-N-T-L-L-G-S-I-H-T-F-D-P-E-A-A-C-D-D(S-T-F-Q-P-C-S-P-R) (273)	6	12		identical to 7-2
	3	28	N-G-D-T(S-L-L-S-T-I-E-N-Y-I-S-A-Q-A-I-V-Q-G-I-S-N-P-S-G-D- L-S-S-G-A-G-L-G-E-P-K) (108)	7	13		identical to 7-3
6	1	10	A-L(A-N-H-K) (279)	8	19		Y-P-E(D-T-Y-Y-N-G-N-P-W) (317)
	2	10	S-D-G-E-Q(L-S-A-R) (413)	9	20		(D-L-T-W) (417)
	3	11	D-G(P-A-L-R) (131)	10	20		(S-Y-A-A-L-L) (423) and 8-8
	4	13	E-V-V(D-S-F-R) (286)	11	21		(D-L-T-W-S-Y) (419) and 8-8
	5	13	A-L-A-N-H(K-E-V-V-D-S-F-R) (286)	12	23		identical to C1-19
	6	14	D-S-G(L-V-L-K) (61)	13	25		A-T-A(M-I-G-F-G-Q-W-L) (142)
	7	15	A-L-Y-S-D(A-A-T-G-T-Y) (363)	14	29		identical to 7-10
	8	16	D-S-W-L(S-N-E-A-T-V-A-R) (15)	15	30		identical to 8-14
	9	18	S-S-S-S-T-Y(S-S-I-V-D-A-V-K) (378)	C1	1	0	(D-S-S-R) (241)
	10	20	S-I-Y-T-L-N-D-G-L(S-D-S-E-A-V-A-V-G-R) (305)	2	3		(N-D-L) (163) & (S-Y) (165)
	11	22	identical to 5-2	3	4		(Q-H-R) (194)
	12	24	(T-L-V-D-L-F-R) (68)	4	9		(N-Q-T-G-Y) (175)
	13	26	Q-G-S-L-E(V-T-D-V-S-L-D-F-F-K) (352)	5	10		(C-Y) (223)
	14	27	L-L-D-N-G-Y-T(S-T-A-T-D-I-V-W-P-L-V-R) (160)	6	11		T-I(A-V-Q-H-R) (194) & (V-A-Q-Y) (169)
	15	32	A-T-A-M-I-G-F-G-Q-W-L-L-D(N-G-Y-T-S-T-A-T-D-I-V-W-P-L-V-R) (160)	7	11		(W-N-Q-T-G-Y) (175)
	15	32	T-A-I-L-N-N-I-G-A-D-G-A-W-V-S-G-A-D(S-G-I-V-V-A-S-P-S-T-D- N-P-D-Y-F-Y-T-W-T-R) (54)	8	12		(T-G-S-F) (232)
	16	33	Y-P-E-D-T-Y-Y-N-G-N-P-W-F-L-C-T-L-A-A-A-E-Q(L-Y-D-A-L-Y-Q-W-D- K) (337)	9	13		identical to C1-8
7	1	9	(G-R-P-Q-R) (125)	10	14		(N-D-L-S-Y) (165)
	2	10	(Q-W-D-K) (337)	11	14		N-D(L-S-Y-V-A) (167)
	3	10	(T-W-T-R) (54)	12	15		I-L-A-N-F(D-S-S-R) (241)
	4	12	T-G(S-W-G-R-P-Q-R) (125)	13	16		A-L-V(E-G-S-A-F) (202)
	5	15	identical to 6-7	14	16		A-T-A-V-G(S-S-C-S-W-C-D-S-Q-A-P-E-I-L-C) (222)
	6	16	F-N-V-D-E(T-A-Y-T-G-S-W) (120)	15	17		A-T-A-V(G-S-S-C-S-W-C-D-S-Q-A-P-E-I-L-C-Y) (223)
	7	17	F-N-V-D-E-T-A-Y(T-G-S-W-G-R-P-Q-R) (125)	16	18		(I-L-A-N-F) (237)
	8	18	F-N-V-D-E-T-A-Y(T-G-S-W-G-R) (122)	17	19		identical to C1-16
	9	25	A-T-L-D(S-W-L-S-N-E-A-T-V-A-R) (15)	18	19		identical to C1-16
	10	29	D-L-T-W(S-Y-A-A-L-L) (423)	19	22		(L-Q-S-F-W) (228)
	11	31	Y-P-E-D(T-Y-Y-N-G-N-P-W-F-L-C-T-L-A-A-A-E-Q-L-Y) (329)	20	26		D-L-W-E-E(V-N-G-S-S-F-F) (187)
8	1	0	(S-G-K) (244)	21	28		D-L-W-E(E-V-N-G-S-S-F) (186)
	2	4	(T-A-N-N-R-R) (429)	22	30		A-T-A-V(G-S-S-C-S-W-C-D-S-Q-A-P-E-I-L-C-Y-L-Q- S-F-W-T-G-S-F-I-L-A-N-F) (237)
	3	5	(P-Q-R) (125)	23	31		A-T(A-V-G-S-S-C-S-W-C-D-S-G-A-P-E-I-L-C-Y-L-Q- S-F-W-T-G-S-F-I-L-A-N-F-D-S-S-R) (241)
	4	9	identical to 7-1				

fragment while negligible differences were observed in the oligosaccharide contents of the two different G1 preparations. The extent of glycosylation seems thus to vary between batches of AMG 200 L glucoamylase and the present evidence for 34 substituents in the glycosylated region is in good agreement with complete glycosylation of approximately 35 O-glycosylated positions estimated previously by reductive β -elimination and peptide sequencing [5, 9].

In the present study the monosaccharide and oligosaccharide compositions of the G1 and G2 E1 fragments matched very well. It appears reasonable therefore to conclude that G2 is a degradation product of G1. The unique capacity of G1 to bind onto and to digest raw starch granules [3, 8] seems thus associated with the COOH-terminal segment which is absent in G2. Supporting evidence is that the oxidation of two tryptophans, Trp⁵⁹⁰ and Trp⁶¹⁵ near the COOH-terminus of G1 is accompanied by loss of raw starch binding capacity (B. Svensson, unpublished observations) without significant loss of activity towards soluble substrates [25, 26]. Moreover, a glycopeptide, isolated from *A. niger* glucoamylase preparations, which was similar in size and amino acid composition to the COOH-terminal region of G1, was recently found to inhibit the adsorption of G1 onto starch granules [27]. However, the structural requirements for the raw starch binding and digestion of G1 have not yet been elucidated.

Boel et al. [6] found a single glucoamylase gene in the genome of *A. niger* and suggested that a G2 form of *A. niger* glucoamylase was coded for by an observed unique G2 mRNA derived from G1 mRNA by differential splicing. The presumptive protein product would be similar in size to the present G2, but its sequence of an eight-residue-long COOH-terminal segment would diverge from the known structure of glucoamylase:

from protein [5] and G1 mRNA [6] sequences

-Ser-Thr-Ser-Ser-Thr-Ser-Cys-Thr⁵¹⁰-

from the presumptive G2 mRNA [6] sequence

-Thr-Thr-Arg-Ser-Gly-Met-Ser-Leu-COOH.

Since in the present study three types of COOH-terminal fragments, CB3-G2, E1-G2 and (T1-2)-G2, were isolated in high yields by different techniques and contained neither homoserine/methionine nor additional arginine and leucine residues it is unlikely that the hypothetical G2 form exists in significant amounts in our G2 preparation. It is also unlikely that this G2 form deduced from the reported G2 mRNA [6], if present in significant amounts in the commercial enzyme preparations, was not detected during the enzyme purification [8]. On the other hand, we cannot exclude the possibility that *A. niger* produces multiple forms via either limited proteolysis of the G1 protein or differential splicing of glucoamylase mRNA [6] depending on the growth conditions. However, so far we have only been able to demonstrate the existence of the former type of G2 protein and the fate of a possible differentially spliced mRNA and its predicted translation product remains unclear at the moment.

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