Substitution of asparagine residues in *Aspergillus awamori* glucoamylase by site-directed mutagenesis to eliminate N-glycosylation and inactivation by deamidation

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Aspergillus awamori glucoamylase is a secreted glycoprotein containing N-linked carbohydrate recognition sites at Asn-171, Asn-182 and Asn-395. Site-directed mutagenesis was performed at Asn-182 and Asn-395 to determine whether these residues were N-glycosylated by *Saccharomyces cerevisiae*, to investigate the function of any glycans linked to them, and to determine the effect of their deamidation on glucoamylase thermostability. Asn-171 and Asn-395, but not Asn-182, were N-glycosylated. Deletion of the glycan N-linked to Asn-395 did not affect specific activity, but greatly decreased enzyme secretion and thermostability. The mutant lacking the N-glycan linked to Asn-395 was synthesized very slowly, and was more associated with cell membrane components and susceptible to proteinase degradation than were wild-type or other mutant glucoamylases. Its secreted form was 30-fold less thermostable than wild-type enzyme at pH 4.5. Replacement of Asn-182 by Gln to eliminate deamidation at this site did not change glucoamylase specific activity or thermostability, while replacement by Asp decreased specific activity about 25%, but increased thermostability moderately at pH 4.5 below 70 °C. Both mutations of Asn-182 increased glucoamylase production.

INTRODUCTION

Glucoamylase (GA; 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3), which cleaves glucose from the non-reducing ends of starch and related oligosaccharides, is used commercially to produce high-glucose syrup to be made into sweeteners and ethanol. GA from *Aspergillus niger* and *Aspergillus awamori*, whose primary structures are identical (Svensson et al., 1983; Nunburg et al., 1984), is a secreted glycoprotein containing both O- and N-glycosidically linked carbohydrates. The former are clustered within a heavily glycosylated segment of 72 amino acids linking the catalytic (residues 1–440) and starch-binding domains (residues 513–616) (Gunnarsson et al., 1984). The crystal structure of the first 470 residues of the closely related *A. awamori* var. *X100* GA showed that the first part of the O-glycosylated segment is in an extended belt conformation, wrapping around the catalytic domain (Aleshin et al., 1992). Through selected deletions, Evans et al. (1990) and Libby and co-workers (C. B. Libby, C. A. G. Cornett, C. Ford and P. J. Reilly, unpublished work) found that this region facilitated secretion and increased thermostability, but did not bind starch granules or affect activity. *A. niger*/*A. awamori* GA has three Asn-Xaa-Ser/Thr carbohydrate-recognition sequences, Xaa being any amino acid except proline (Gavel and Heijne, 1990; Berg and Grinnell, 1993), with bulky N-linked carbohydrate groups attached to Asn-171 and Asn-395, but not to Asn-182 (Svensson et al., 1983; Aleshin et al., 1992). In the GA crystal structure the two chains are on either side of the active site. The function of N-glycosylation in GA is unknown, although it directs correct folding (Gibson et al., 1980; Dube et al., 1988; Li et al., 1993), facilitates secretion (Dube et al., 1988; Bocchinfuso et al., 1992; Delorme et al., 1992), and enhances thermostability (Olsen and Thomsen, 1991; Joao et al., 1992) of other proteins. In general, bound carbohydrate stabilizes GA, because at high temperatures chemically deglycosylated GA decays faster than native GA (Pazur et al., 1970; Shenoy et al., 1984).

The GA gene from *A. awamori* has been cloned and expressed in *Saccharomyces cerevisiae* (Innis et al., 1985). O-glycosylation is more extensive in this form than in native GA (Innis et al., 1985). However, until this project it was not known whether Asn-171, Asn-182, and Asn-395 were N-glycosylated by *S. cerevisiae*.

Both Asn-182 and Asn-395 are part of Asn-Gly sequences whose Asn residues are very susceptible to non-catalytic deamidation reactions (Robison and Rudd, 1974; Tyler-Cross and Schirch, 1991; Wright, 1991). Site-directed mutagenesis of Asn-182 to Ala-182 in *A. awamori* GA expressed in *S. cerevisiae* (Sierks et al., 1993) removed the labile Asn-182–Gly-183 sequence and markedly improved enzyme thermostability at pH 4.5 up to 70 °C, implying that deamidation of Asn-182 could be one of the major thermoinactivation pathways of GA under these conditions (Chen et al., 1994). The mutation failed to stabilize the enzyme above 70 °C, because unfolding followed by irreversible formation of incorrect structures started to become the fastest inactivation pathway. The mutation also caused a moderate decrease in enzyme activity (Sierks et al., 1993), but yielded more secreted enzyme (Chen et al., 1994). Another mutation of this residue, Asn-182 → Asp (Bakir et al., 1993), also caused a moderate loss of activity. Secretion and thermostability of this mutant enzyme were not tested. Mutations of Asn-395 have not been reported. The work reported here had three goals: (1) to determine which of Asn-171, Asn-182 and Asn-395 were glycosylated by *S. cerevisiae*; (2) to study the effect of removing the possible N-glycosylation sites at Asn-182 and Asn-395 on GA secretion, specific activity and thermostability; (3) to study the effect on GA thermostability of eliminating possible deamidation

Abbreviations used: GA, glucoamylase; GAS and GAS′, the larger and smaller degraded forms of glucoamylase digested by subtilisin; PNGase F, peptide-N-glycosidase F; PMSF, phenylmethanesulphonyl fluoride; WT, wild-type.

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sites at Asn-182 and Asn-395. To do this, Asn-182 and Asn-395 were substituted with Gln and Asn-182 → Asp, Asn-182 → Gln, and Asn-395 → Gln GAs were produced and characterized.

EXPERIMENTAL

Materials

S. cerevisiae strain C468 (α *leu2-3 leu2-112 his3-11 his3-15 mal*$^-$) and its expression plasmids containing the *A. awamori* WT GA gene, YEpGAC9 and YEpPM18, were gifts from Cetus Corporation. The *S. cerevisiae* strain containing Asn-182 → Asp mutant GA gene was provided by Dr. U. Bakir. All restriction enzymes were obtained from Promega. Acarbose was a gift from Miles Laboratories, subtilisin was obtained from Fluka, and peptide-N-glycosidase F (PNGase F) was from Oxford GlycoSystems.

Site-directed mutagenesis

A 1.77 kb *Xho*I-*Bam*HI fragment of the *A. awamori* gene in the YEpPM18 plasmid was subcloned into a phagmid vector, pGEM-7Zf(+) from Promega. Site-directed mutagenesis was performed by using a Muta-Gene phagmid *in vitro* mutagenesis kit from Bio-Rad, which is based on a method developed by Kunkel et al. (1987). Synthetic oligonucleotides GGGAAGAAGTCCAAGGCTCGTCTTTC and CACGCCGCAAGCCAAGGCTCCATGTC, designed to replace the Asn codons with Gln codons (underlined) at AA positions 182 and 395 respectively were used as primers to carry out the reactions. After extension and ligation, the reaction products were used to transform *Escherichia coli* strain MV1190. The resulting colonies were screened by using colony hybridization techniques with an oligonucleotide probe labelled non-radioactively using a Genius kit from Boehringer Mannheim. The mutations were further verified by DNA sequencing before subcloning them into the expression vector YEpPM18. Mutated plasmids were transformed into *S. cerevisiae* competent cells prepared by using lithium acetate (Ito et al., 1983). Transformants were selected by growth on SD-His minimal medium that did not contain leucine (Innis et al., 1985).

Production and purification of GA

Wild-type (WT) GA expressed in YEpGAC9 was produced and purified previously (Chen et al., 1994). WT and mutated GAs expressed in YEpPM18 were produced in 10 litre batches in a 15 litre fermentor over 72 h at 30 °C and pH 4.5 (Chen et al., 1994). Broth supernatant was concentrated 50-fold and diafiltered with 0.5 M NaCl/0.1 M sodium acetate buffer at pH 4.4 with a 10 kDa cutoff Amicon S1 spiral ultrafiltration cartridge. Half the concentrate at a time was loaded on to a pre-equilibrated 10 mm internal diameter × 64 mm long column containing acarbose–Sepharose gel, prepared by activating Pharmacia LKB Sepharose CL 6B gel with butane-1,4-diol diglycidyl ether and then coupling it with acarbose. Bound enzyme was eluted with 1.7 M Tris buffer at pH 7.6. The eluate was dialysed against water and recovered by freeze-drying.

Assays

Protein concentration was determined with the Pierce bicinchoninic acid protein assay (Smith et al., 1985). Activities of WT and mutated GAs were determined with 4% maltose as substrate in 0.05 M sodium acetate buffer at pH 4.5 and 50 °C. The glucose liberated from six samples at different incubation times in the linear region was detected by a glucose oxidase/*o*-dianisidine colorimetric assay (Banks and Greenwood, 1971). One unit is the amount of enzyme required to produce 1 μmol/min glucose under the conditions of the assay. To measure intra- and extracellular GA activities, yeast cells with different GA genes were grown in SD-His medium in shake-flasks at 30 °C for 2–5 days with no pH control. The cell density was determined at 600 nm using a 10 mm pathlength cuvette. The supernatants of 16 ml culture samples were concentrated 20-fold, diafiltered with 0.05 M sodium acetate buffer at pH 4.5 by using Amicon Centricon-30 microconcentrators and assayed. The pelleted cells were washed with deionized water, resuspended in lysis buffer [50 mM Tris (pH 7.5)/1% sodium deoxycholate/1% Triton X-100/0.1% SDS/1 mM phenylmethanesulphonyl fluoride (PMSF)] and vortex-mixed with 426–600 μm-diameter acid-washed glass beads (Jazwinski, 1990). Soluble cell extract was separated from insoluble cell debris by centrifugation, and each fraction was assayed for activity or subjected to immunoblotting.

Electrophoresis and immunoblotting

Soluble intra- and extra-cellular fractions of 5-day-old yeast cultures prepared as above were added to 3.5-fold-concentrated SDS/PAGE sample buffer and heated in boiling water for 3 min. Soluble intra- (10%) and extra-cellular fractions (1%) were subjected to SDS/PAGE on a 1-mm-thick 4–20% gel. Proteins were then transferred electrophoretically from the gel on to a 0.2 μm nitrocellulose membrane with a Bio-Rad Trans-Blot SD semi-dry transfer cell. The resulting Western blot was incubated with 10% Kinkegard and Perry milk diluent as the blocking solution and probed with polyclonal antibodies raised against *A. niger* GA. The immunoreactive proteins were detected with Bio-Rad Protein A colloidal gold, followed by silver enhancement.

Determination of N-glycosylation sites in GA

Purified WT and mutant GAs, 2 mg/ml in 0.05 M phosphate buffer at pH 6.8, were partially digested with 0.2 mg/ml subtilisin at room temperature for 24 h. To inactivate subtilisin, PMSF was added to a final concentration of 10 mM. Subtilisin digestion produced two major forms of the enzyme, GAS and the smaller GAS′. A DEAE Acti-Disk cartridge from FMC was used to separate subtilisin and GAS from GAS′ and uncleaved GA, with the first two freely passing through the cartridge while in 0.01 M phosphate loading buffer at pH 6.8. GA and GAS′ were eluted with a 0–0.4 M NaCl step salt gradient in the same buffer. The eluted fractions containing GAS′ were pooled, concentrated 20-fold, and diafiltered with 0.05 M phosphate buffer at pH 6.8. The concentrated samples, containing 5–10 μg of GAS′, were added to an equal volume of denaturation buffer [20 mM phosphate buffer (pH 7.5)/50 mM EDTA/0.5% SDS/1% β-mercaptoethanol/1% Nonidet P40], boiled at 100 °C for 3 min, and partially de-N-glycosylated with 1 unit of PNGase F in the reaction buffer (50 mM EDTA/0.02% sodium azide/50% glycerol in 20 mM phosphate buffer, pH 7.5) for 0.5–18 h. The reaction mixture was then subjected to discontinuous SDS/PAGE on a 0.75-mm-thick 7.5% gel to determine the number of de-N-glycosylated forms.

Irreversible thermoinactivation of GA

Purified WT and mutant GAs, 80 μg/ml in 0.05 M sodium

acetate buffer at pH 3.5, 4.5 or 5.5, were incubated at six different temperatures over 2.5 °C intervals from 65 °C to 77.5 °C (five temperatures from 60 °C to 70 °C for Asn-395 → Gln GA). Six samples were removed periodically from each, promptly chilled in an ice bath, and assayed for GA activity after 24 h. The irreversible thermoinactivation process obeyed first-order kinetics, so its rate coefficient, k_d, was determined by linear regression in semilogarithmic co-ordinates.

RESULTS

Production and purification of GA

Yeast cells carrying YEpAC1 (lacking a GA gene), YEpPM18 (bearing a WT GA gene), and mutated YEpPM18 plasmids (bearing Asn-182 → Asp, Asn-182 → Gln, or Asn-395 → Gln GA genes) were grown on SD-His agar plates containing 1 % soluble starch. No clear halo occurred around colonies carrying YEpAC1, because no GA was produced to digest soluble starch (Figure 1). The largest halo was around WT yeast colonies, while the smallest was around colonies containing Asn-395 → Gln GA.

Similar growth curves (Figure 2) and glucose utilization rates were observed in each fermentation to produce GA. No loss of activity was observed for any mutant GA on acarbose–Sepharose affinity chromatography. After purification, Asn-182 → Gln and Asn-395 → Gln mutant GAs had specific activities similar to that of WT GA, while the specific activity of Asn-182 → Asp GA was about 25 % less (Table 1). The total purified GA recovered from the fermentation runs decreased in the order Asn-182 → Asp > WT ≈ Asn-182 → Gln > Asn-395 → Gln, indicating that the production of the first was greatly improved by the presence of Asp at position 182, while the production of the last was severely impeded by removal of the N-glycan linked to the Asn-395 residue.

Localization of GA

To study the secretion of WT and mutant GAs, intra- and extracellular fractions of shake-flask yeast cultures bearing different GA genes were prepared. Different cultures had similar growth patterns (Figure 2). Purified forms of all enzymes were judged to bind equally to polyclonal antibodies raised against *A. niger* GA (results not shown). Immunoactivities of soluble intracellular fractions of WT, Asn-182 → Gln and Asn-182 → Asp GAs were about equal to each other, as were those of their extracellular fractions, and in each case they were much higher than immunoactivities of intra- and extra-cellular fractions of Asn-395 → Gln GA (Figure 3).

In addition, enzymic activities of GAs in different parts of the cell were measured (Table 2). Intra- and extra-cellular GA activities of a 5-day culture each decreased in the order Asn-182 → Asp > Asn-182 → Gln > WT ⩾ Asn-395 → Gln, with approx. 90 % of the activity of the first three enzymes and only 60 % of the activity of the last secreted to the culture supernatant. Most of the unsecreted active GA was bound to cell debris, with very little in the soluble lysate. The activity of Asn-395 → Gln GA was very low in each cell fraction compared with other GAs. The low levels of both immuno- and enzyme activities of Asn-395 → Gln GA suggests that this mutant was either synthesized very slowly or was subject to rapid proteolytic degradation or instability. Production of WT GA into the shake-flask supernatant virtually stopped after three days from the time that the cells stopped growing, while Asn-182 → Gln and Asn-182 → Asp GA levels continued to rise for two more days (Figure 4). Asn-395 → Gln

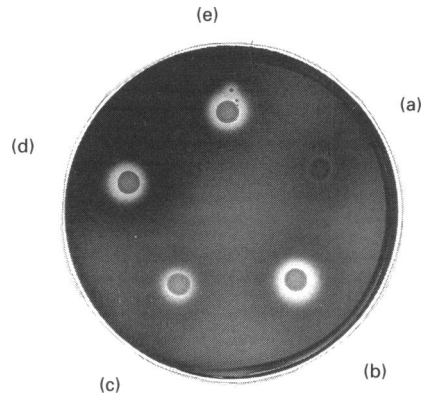

Figure 1 Starch-clearing plate assay for GA expression

S. cerevisiae cells in 5 μl (A_{600} = 1) carrying YEpAC1 (a), YEpPM18/WT (b), YEpPM18/ Asn-395 → Gln (c), YEpPM18/Asn-182 → Asp (d) and YEpPM18/Asn-182 → Gln (e) plasmids were grown on SD-His plates containing 1 % soluble starch at 30 °C for 3 days and then overnight at 50 °C. Plates were stained with iodine vapour to observe clear halos produced around yeast colonies.

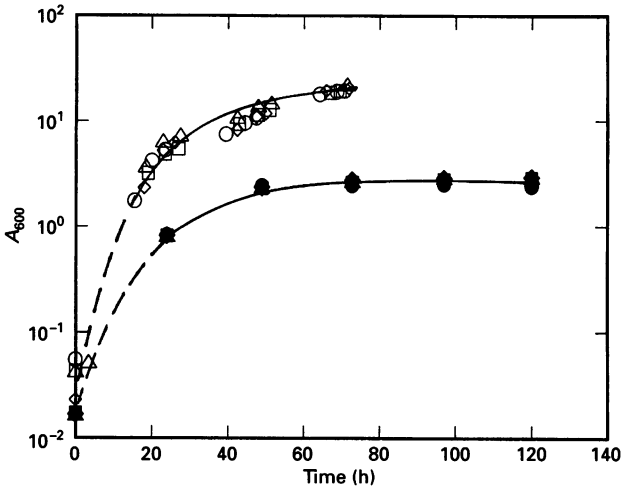

Figure 2 Growth of *S. cerevisiae* in shake-flask and fermentation cultures

Yeast cells carrying WT (○,●), Asn182 → Asp (□,■), Asn-182 → Gln (◇,◆), or Asn-395 → Gln (△,▲) GA genes were grown either in 10-litre batches in a 15-litre fermentor (open symbols) or in 250-ml shake-flasks (closed symbols). Growth conditions were as described in the Experimental section.

Table 1 Specific activity and total protein after purification of GAs produced by batch fermentation of *S. cerevisiae* at 30 °C and pH 4.5 for 72 h

GA form	Protein (mg)	Specific activity (units/mg)
WT	68.1	18.9 ± 0.7*
Asn-182 → Asp	113	13.8 ± 0.6
Asn-182 → Gln	62.5	17.8 ± 0.8
Asn-395 → Gln	29.1†	19.3 ± 0.7

* Results are means ± S.D.
† Average for two batches.

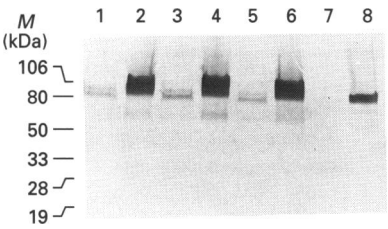

Figure 3 Localization of soluble GA

Soluble intra- (lanes 1, 3, 5 and 7) and extra-cellular (lanes 2, 4, 6, and 8) fractions of *S. cerevisiae* carrying WT (lanes 1 and 2), Asn-182 → Gln (lanes 3 and 4), Asn-182 → Asp (lanes 5 and 6) or Asn-395 → Gln (lanes 7 and 8) GA genes were prepared and subjected to Western blotting as described in the Experimental section. Molecular-mass (*M*) markers (kDa) are located to the left.

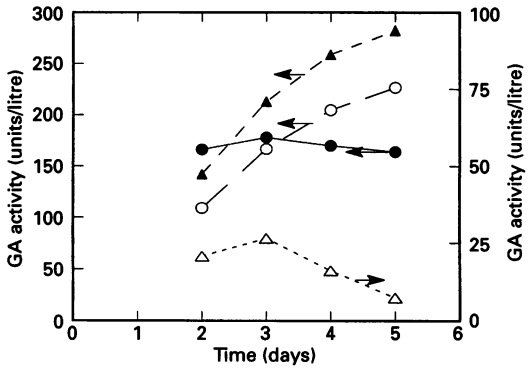

Figure 4 Production of *A. awamori* GA from *S. cerevisiae*

S. cerevisiae cells carrying WT (●), Asn-182 → Gln (○), Asn-182 → Asp (▲), or Asn-395 → Gln (△) GA genes were grown in shake-flasks at 30 °C without pH control for 2–5 days. Clear supernatant was prepared and assayed for GA activity as described in the Experimental section.

Table 2 GA activity (units/litre) in supernatant and cell lysate of 5-day cultures of *S. cerevisiae* bearing WT or mutant GA genes

		Intracellular	
GA form	Extracellular	Soluble	Insoluble
WT	164	0.6	16.7
Asn-182 → Asp	282	0.9	39.1
Asn-182 → Gln	226	1.0	27.4
Asn-395 → Gln	7.4	0.1	5.7

GA activity in the supernatant decreased very sharply after 3 days, suggesting proteolytic degradation or enzyme instability.

To study the proteinase resistance of WT and mutant GAs, purified GA preparations were digested by subtilisin. The SDS/PAGE patterns of digested fractions of WT and Asn-395 → Gln GAs were very similar when PMSF was added to the electrophoretic samples to inactivate subtilisin just before denaturation

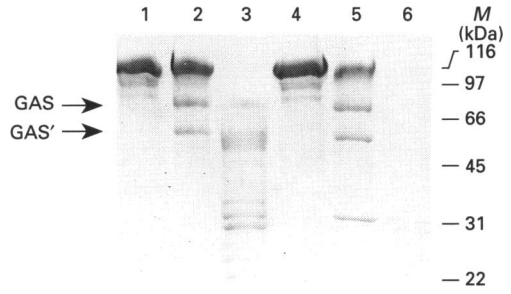

Figure 5 Subtilisin digestion of *A. awamori* GA expressed in *S. cerevisiae*

WT (lanes 1–3) and Asn-395 → Gln (lanes 4–6) GAs were digested with subtilisin for 24 h and subsequently analysed by SDS/4–15%-PAGE in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of PMSF. Lanes 1 and 4 represent undigested GAs. Each lane was loaded with the equivalent of 20 μg of undigested GA. Positions of GAS and GAS′ forms of degraded GAs and molecular-mass (*M*) markers (kDa) are located to the left and right respectively.

by boiling (Figure 5). GAS and GAS′, the two major forms of degraded GA, had specific activities similar to the parent form, with apparent molecular masses of about 80 and 60 kDa respectively. This pattern was somewhat different from those of *A. niger* GA digested by subtilisin from different sources (Stoffer et al., 1993). However, without PMSF addition, Asn-395 → Gln GA and its partially degraded forms were more susceptible to further digestion by subtilisin than were equivalent forms of WT GA during heat treatment (Figure 5). The SDS/PAGE electrophoretic patterns of Asn-182 → Asp and Asn-182 → Gln GAs digested by subtilisin were very similar to that of WT GA, and their parent and degraded forms were equally resistant to further digestion by subtilisin (results not shown). This suggests that Asn-395 → Gln GA may be very susceptible to intracellular proteinase digestion, resulting in its low net production.

Determination of N-glycosylation sites in GA

PNGase F, which specifically hydrolyses N-glycosidic bonds between N-glycans and Asn residues in proteins, was used to determine N-glycosylation sites in GA, based on the mobility difference between untreated and partially de- N-glycosylated forms of protein on SDS/PAGE (Alexander and Elder, 1989). However, because GA was too large to clearly show mobility differences on SDS/PAGE, GAS′ was used instead. The latter likely has all three putative N-glycosylation sites in GA, since (1) the *A. awamori* var. *X100* GA crystal structure suggests that Glu-400 is the catalytic base (Harris et al., 1993), so GAS′, with a specific activity similar to untreated GA, has at least the first 400 residues of GA; and (2) more than 416 N-terminal residues are required for a genetically truncated *A. awamori* GA to be active (Evans et al., 1990). Two de-N-glycosylated forms were produced from WT GAS′ by PNGase F (Figure 6a), indicating that two out of three potential N-glycosylation sites in *A. awamori* GA were N-glycosylated by *S. cerevisiae*. Two de-N-glycosylated forms were also observed for Asn-182 → Gln GAS′ (Figure 6b), while only one form was observed for Asn-395 → Gln GAS′ (Figure 6c), suggesting that Asn-171 and Asn-395 were N-glycosylated by *S. cerevisiae*, while Asn-182 was not. This N-glycosylation pattern is the same as those of *A. niger* native GA (Svensson et al., 1983) and *A. awamori* var. *X100* native GA (Aleshin et al., 1992).

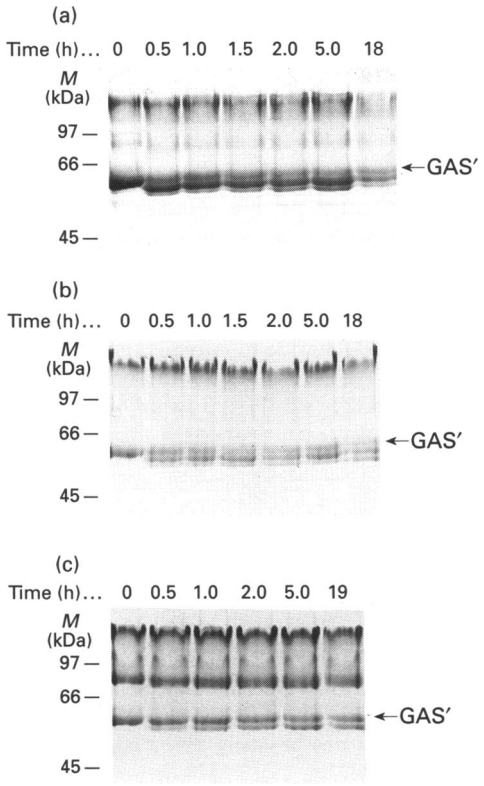

Figure 6 Partial de-N-glycosylation of GAS′

GAS′ degraded from WT (a), Asn-182 → Gln (b) and Asn-395 → Gln (c) GAs by subtilisin was partially de-N-glycosylated with PNGase F for given times and analysed by SDS/7.5%-PAGE. Molecular-mass (*M*) markers (kDa) are located to the left.

Table 3 First-order thermoinactivation coefficients (k_d, s^{-1}) of WT and mutant GAs at 70 °C and different pH values

		Thermoinactivation coefficient (k_d, s^{-1})		
GA form	pH ...	3.5	4.5	5.5
WT		0.0033 ± 0.0001*	0.0023 ± 0.0001	0.0022 ± 0.0001
Asn-182 → Asp		0.0036 ± 0.0001	0.0016 ± 0.0001	0.0023 ± 0.0002
Asn-182 → Gln		0.0032 ± 0.0002	0.0017 ± 0.0002	0.0020 ± 0.0002
Asn-395 → Gln		0.1260 ± ND†	0.0765 ± 0.0082	0.0150 ± 0.0007

* Results are means ± S.D.
† Not determined.

Irreversible thermoinactivation of GA

Irreversible thermoinactivation of all GAs in buffer was studied at 70 °C and pH values from 3.5 to 5.5, k_d values being shown in Table 3. WT, Asn-182 → Asp, and Asn-182 → Gln GAs had roughly equal stabilities, and were much more stable than Asn-395 → Gln GA throughout this pH range. Asn-182 → Asp and Asn-182 → Gln GAs were somewhat more thermostable than WT GA at pH 4.5, but not at pH 3.5 or 5.5. Asn-395 → Gln GA was most stable at pH 5.5, with 33-fold and 7-fold less stability than WT GA at pH 4.5 and 5.5 respectively. However, no decay

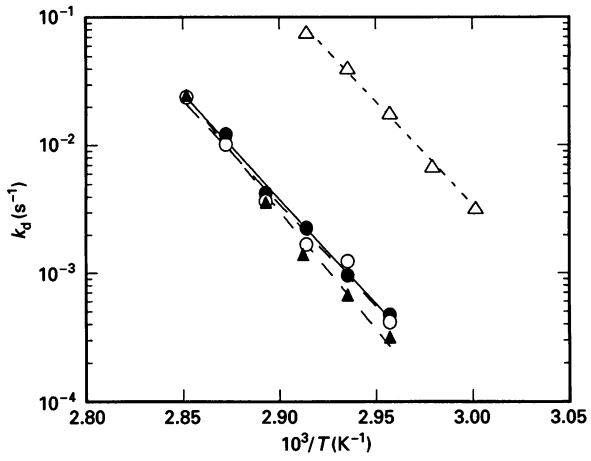

Figure 7 Effect of temperature on GA thermoinactivation

First-order inactivation coefficients (k_d) of WT (●), Asn-182 → Gln (○), Asn-182 → Asp (▲) or Asn-395 → Gln (△) GAs were measured in pH 4.5 buffer at indicated temperatures as described in the Experimental section.

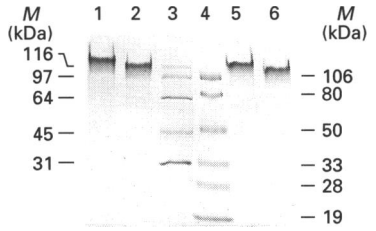

Figure 8 SDS/PAGE mobility of regular and de- N-glycosylated WT GAs

WT GAs expressed by YEpPM18 (lanes 1 and 2) and YEpGAC9 (lanes 5 and 6) were de-N-glycosylated (lanes 2 and 6) with PNGase F at 37 °C for 18 h and analysed by SDS/PAGE on an 1-mm-thick 4–20% gradient gel. Markers in lanes 3 and 4 have their molecular masses (*M*) (kDa) indicated to the left and right respectively.

in Asn-395 → Gln GA activity occurred at 4 °C in 0.05 M sodium acetate buffer at pH 4.5 for several weeks, or at 50 °C with 4% maltose in the same buffer for more than 1 h.

The effect of temperature on k_d values of different GAs in pH 4.5 buffer is shown in Figure 7. Unlike WT GA expressed in a previous study by the low-expression plasmid YEpGAC9 (Chen et al., 1994), the slope in the Arrhenius plot of WT GA expressed in this study by the high-expression plasmid YEpPM18 was constant across the temperature range tested. YEpPM18 GA was more thermostable than YEpGAC9 GA below 70 °C, presumably because of its more extensive O-glycosylation, exhibited by the slightly lower mobilities on SDS/PAGE of both parent and de- N-glycosylated forms of YEpPM18 GA compared with YEpGAC9 GA (Figure 8). Asn-182 → Gln GA was as thermostable as WT GA, while Asn-182 → Asp GA was more stable than WT GA below 70 °C. Asn-395 → Gln GA was approx. 30-fold less thermostable than WT GA across the temperature range tested.

Activation enthalpies ($\Delta H^{\ddagger}$), entropies ($\Delta S^{\ddagger}$) and free energies ($\Delta G^{\ddagger}$) for thermoinactivation of different GAs calculated by transition-state theory are shown in Table 4. Values of the first two for WT GA expressed by YEpPM18 were similar to those

Table 4 Activation parameters for thermoinactivation of WT and mutant GAs at pH 4.5 and 65 °C

GA form	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol·K)	$\Delta G^{\ddagger}$ (kJ/mol)
WT	311±10*	610±29	105
Asn-182 → Asp	352±19	727±54	106
Asn-182 → Gln	305±20	591±59	105
Asn-395 → Gln	304±11	620±32	95

* Results are means±S.E.M.

above 70 °C for WT GA expressed by YEpGAC9 (Chen et al., 1994).

DISCUSSION

We have used site-directed mutagenesis (1) to determine the N-glycosylation sites in *A. awamori* GA expressed by *S. cerevisiae*; (2) to study the function of N-linked oligosaccharides attached to the Asn-395 residue; and (3) to eliminate the possible deamidation site on Asn-182 in an attempt to increase GA thermostability. Mutagenesis provides an effective method to study specifically functions of different residues or glycans in proteins, which is not easily achieved by traditional methods such as chemical or enzymic modification or addition of glycosylation inhibitors. In the work reported here, no difference in occupancy of Asn-Xaa-Ser/Thr N-glycosylation sites was observed among *A. niger* native GA, *A. awamori* var. *X100* native GA and *A. awamori* GA expressed in *S. cerevisiae*. Only Asn-171 and Asn-395 residues were covalently linked to carbohydrate, while Asn-182 was not, indicating that this recognition sequence was not a sufficient condition for N-linked glycosylation. The nitrogen atom on the amide side chain of Asn-182 is not exposed to solvent but is instead turned to the main chain (Aleshin et al., 1992), unlike those of Asn-171 and Asn-395, suggesting that it is inaccessible to oligosaccharyltransferase for core glycosylation, which has been proposed to occur after primary folding of growing polypeptide chains in the endoplasmic reticulum (Hughes, 1983; Bulleid et al., 1992). On the other hand, the relative proximity of Asn-171 and Asn-182 in GA could suggest that the addition of an N-glycan at the former may block subsequent N-glycosylation at the latter, either by steric hindrance or by changing the tertiary structure of GA. Chicken ovalbumin (Sheares, 1988) and β-amyloid precursor protein (Påhlsson et al., 1992), whose tertiary structures are not available, also contain two closely spaced putative N-glycosylation sites, with only the first one occupied. The second site in chicken ovalbumin could be glycosylated *in vitro* when the protein was in a denatured state (Glabe et al., 1980), while deletion of the first site in both proteins by site-directed mutagenesis did not cause the second one to become glycosylated *in vivo*, suggesting that, in those cases, the tertiary structure and accessibility of the recognition sequence for oligosaccharytransferase are important for core glycosylation at specific sites. To understand clearly why Asn-182 is not glycosylated, another mutation to eliminate N-glycosylation at Asn-171 may be necessary.

The presence of oligosaccharide at Asn-395 is important for the biosynthesis, secretion, thermostability and proteinase resistance of GA, but not for its enzymic and immunological activities. The lack of any effect of Asn-395 glycosylation on each of these activities suggests that GA can be correctly folded without the presence of this N-glycan. The impaired secretion and extremely low levels of detectable intracellular and extracellular activities of Asn-395 → Gln GA may result from underglycosylation of GA causing a decrease in the solubility of the translation product, restricting its further assembly and increasing the possibility of proteolytic degradation. Defects in glycosylation that reduce protein solubility and proteinase resistance have been previously reported (Gibson et al., 1980). In addition, the unsymmetrical N-glycan structure of this mutant, with the N-glycan linked to Asn-171 present on one side of the active site and with no N-glycan on the other side, may affect its processing by oligosaccharide-modifying enzymes and obscure its recognition by receptor proteins in the Golgi apparatus, causing it to be captured on the cell membrane or retarding its transportation out of the cell (Alberts et al., 1989). The 30-fold lower thermostability at pH 4.5 of this mutant compared with WT GA suggests that N-glycosylation may stabilize GA conformation at high temperature. Furthermore, hydroxylamine did not cleave *A. niger* GA between Asn-395 and Gly-396 during amino acid sequencing (Svensson et al., 1983), suggesting that the N-glycan on Asn-395 could also protect that residue from deamidation at high temperatures, since these two reactions occur by similar mechanisms (Bornstein and Balian, 1977; Patel and Borchardt, 1990). However, in the present study the effect of this N-glycan on GA thermostability was so great that the effect of deamidation could not be easily observed.

WT GA expressed by YEpPM18 is more extensively O-glycosylated than WT GA expressed by YEpGAC9 (Figure 8), which is more O-glycosylated than native *A. niger* GA (Innis et al., 1985). It is 1.4–2-fold more thermostable than either below 70 °C at pH 4.3–4.5 (Chen et al., 1994; Flory et al., 1994), but the differences of thermostability caused by different levels of O-glycosylation were far smaller than those associated with the change of N-glycosylation caused by the Asn-395 → Gln mutation.

WT and Asn-182 → Gln mutant GAs had higher specific activities than either Asn-182 → Ala (Sierks et al., 1993; Chen et al., 1994) or Asn-182 → Asp mutant GAs, suggesting that the nitrogen atoms on the side chains of Asn-182 and Gln-182 are important for GA activity. The value of $\Delta H^{\ddagger}$ for inactivation for WT GA expressed by YEpPM18 was much higher than that of WT GA expressed by YEpGAC9 below 70 °C (Chen et al., 1994), which was only slightly higher than the $\Delta H^{\ddagger}$ value of the deamidation reaction (Patel and Borchardt, 1990). This suggests that the effect of deamidation on thermoinactivation of YEpPM18 GA is not as important as that on thermoinactivation of YEpGAC9 GA, presumably because more extensive O-glycosylation of the former restricts conformational change of the catalytic domain after deamidation. Like Asn-182 → Ala GA (Chen et al., 1994), Asn-182 → Asp GA was more thermostable than its corresponding WT GA below 70 °C at pH 4.5, while Asn-182 → Gln GA was not. No difference in SDS/PAGE mobility of either parent or de-N-glycosylated forms of WT, Asn-182 → Gln, and Asn-182 → Asp GAs was observed, suggesting that there was no difference in glycosylation among them. Therefore, the deamidation of Asn-182 may still contribute to thermoinactivation of GA expressed from YEpPM18 below 70 °C at pH 4.5, although its contribution would be very small, based on the difference between thermoinactivation of WT and Asn-182 → Asp GAs. Like the Asn-182 → Ala mutation (Chen et al., 1994), the Asn-182 → Asp mutation failed to stabilize GA from thermoinactivation above 70 °C, suggesting that the contribution of deamidation to enzyme inactivation was diminished, with the irreversible formation of incorrect structure after protein

unfolding becoming dominant. A possible explanation for the failure of the Asn-182 → Gln mutation to greatly increase thermostability would be that deamidation of Gln-182 occurred as rapidly as that of Asn-182 under the conditions used for inactivation. Deamidation of Gln occurs in many other proteins, although its frequency is much less than that of Asn deamidation (Wright, 1991).

Production of GA was improved when Asn-182 was replaced with either Gln or Asp, with continuous production of Asn-182 → Gln and Asn-182 → Asp mutants even after production of WT GA had stopped. About 90% of active enzyme was secreted for all three GAs, suggesting that their secretion efficiencies are similar. Therefore differences in production probably resulted from differences in their biosynthesis rates, intracellular stabilities or susceptibilities to proteolysis.

In conclusion, this study has showed that (1) occupancy of the three N-linked glycosylation sites in *A. awamori* GA is the same in *Aspergillus* and *S. cerevisiae* GAs; (2) the N-glycan attached to Asn-395 increases GA secretion, thermostability and proteinase resistance, but does not affect its catalytic or immunological activity; (3) the Asn-182 side-chain amino group affects GA activity; and (4) deamidation of Asn or Gln at position 182 can contribute somewhat to the thermostability of GA at pH 4.5 up to 70 °C, the extent determined by the GA glycosylation level.

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