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Extracellular Proteolytic Processing of *Aspergillus awamori* GAI into GAII is Supported by Physico-Chemical Evidence

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ABSTRACT

The proportion of glucoamylases, GAI and GAII, in the culture supernatant of *Aspergillus awamori* fermentations depends on the medium C/N ratio in such a way that the transformation of GAI into GAII is favored by the existence of a surplus of the carbon source in the growth medium. This condition also favors the appearance of the proteolytic activity. The authors report the observation that the shift in the isoenzyme proportion was concomitant to the peak of proteolytic activity. A peptide that may have resulted from the continuous degradation of the GAI C-terminal peptide, Gp-1, was isolated by gel filtration and purified by reverse-phase chromatography. This peptide matched with the region G^{14}-A^{34} of the substrate-binding domain of GAI, thus reinforcing the hypothesis of the extracellular proteolytic processing of GAI.

Index Entries: *Aspergillus awamori*; glucoamylase; GAI and GAII; glucoamylase isoenzymes processing; isoenzymes extracellular processing; isoenzymes proteolytic processing.

INTRODUCTION

Aspergillus awamori 2.B.361 U2/1 produces two glucoamylase isoenzymes, GAI and GAII, whose proportion in the culture supernatant depends on the medium C/N ratio. GAI is prevalent in carbon limited fermentations (C/N 10), whereas GAII predominates under nitrogen limitation (C/N 26) *(1)*. GAII (54,000 Dalton) differs from GAI (75,000 Dalton) by lacking a C-terminal region of approx 100 amino acids residues *(2)*.

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Because of the specific catalytic properties of the isoenzymes, it would be desirable to elucidate the in vivo mechanism responsible for the generation of the GAII molecule. The limited in vitro degradation of GAI using fungal acid proteases or subtilisin resulted in the production of GAII, by liberating the glycopeptide GpI of GAI *(3)*, suggesting proteolysis as a possible mechanism. A relationship between the conversion of GAI into GAII and the appearance of proteolytic activity during the fermentation was also observed indicating that extracellular proteolysis could be the in vivo mechanism *(4,5)*. This possibility was explored. In consideration of the C/N 26 fermentation where GAII prevails at the end of the cultivation, the isoenzyme profile was also determined at its early stages where no significant proteolytic activity would be expected, and therefore GAI would be the predominant molecular species. For this purpose, samples were collected at selected time intervals during the course of the whole fermentation. These samples were analysed for isoenzyme composition and the presence of peptides derived from the C-terminal region of GAI. For comparison, the same procedure was carried out for C/N 10 fermentations, in which no shift in the pattern of the isoenzymes would be expected. In both cases, the samples were also used for glucoamylase and protease activity determination.

The prevalence of GAI or GAII in the corresponding C/N 10 or C/N 26 fermentations was confirmed in this work by comparing the profiles of the peptides that were obtained after enzymatic cleavage with endoproteinase Lys-C of the reduced and S-alkylated isoenzymes (GAI-RCM and GAII-RCM). The reverse-phase chromatography profiles of the peptides were similar, except for a single peptide that was only present in the digest of GAI-RCM. This mismatched peptide was purified and submitted to amino acid analysis. Its composition was very similar to that of a peptide (residues 554–576) that was expected from the theoretical enzymatic cleavage in the C-terminal region of the GAI isoenzyme.

MATERIAL AND METHODS

Culture and Maintenance, Propagation, and *Aspergillus awamori* Fermentations

All procedures were carried out according to previous work which the same strain, *Aspergillus awamori* 2.B.361.U2/1 was used *(1,6,7)*. During the fermentations, samples were collected and their supernatants were used for glucoamylase and protease activity determination, glucose consumption, GAI and GAII isoenzymes purification and identification, and peptide screening when appropriate.

Crude Glucoamylase and Peptide Isolation

Samples from the culture supernatants were chromatographed in a Biogel P-10 column (120 × 2.5 cm). The elution was performed with

0.5 m*M* acetic acid using a flow rate of 30 mL/h. The effluent was detected by 280-nm absorption and the relevant fractions lyophilized or dried in SpeedVac system (Savant, Farmingdale, NY).

Glucoamylase Purification by Ion-Exchange Chromatography

The crude glucoamylase activity obtained in the void volume of gel permeation were pooled and chromatographed on an anion-exchange column (Pharmacia, FPLC Mono Q, HR 10/10). The elution was performed at a flow rate of 0.7 mL/min with 5 min of 50 m*M* Tris-HCl, pH 8.0 followed by a NaCl gradient (0–0.6 *M*) in the same buffer. The two forms of glucoamylase were detected by a photo dyode array HPLC detector (Waters, Milford, MA) at 215 and 280 nm. The peaks eluting at retention times 21.5 min and 25.2 min were collected, desalted by chromatography on Sephadex G-25 fine column, and dried with a SpeedVac.

Denaturation and S-Carboxymethylation

Both glucoamylase isoenzymes (3 mg) were denaturated in 1 mL of 6 *M* guanidine hydrocloride containing 1 *M* Tris-HCl buffer, pH 8.0, and 10 m*M* EDTA for 16 h at 40°C. After reduction with 1.0 mg of DTT for 4 h at 40°C, the two proteins reacted with 1.5 mg of iodoacetamide for 3 h at 40°C. The reaction was stopped by addition of glacial acetic acid to pH 4.0. The mixture was filtered through a Sephadex G-25 fine column (40 × 1.5 cm) eluted with 50 m*M* amonium hydroxide. The reduced and S-carboxymethylated glucoamylase isoenzymes GAI-RCM and GAII-RCM were detected by absorbance at 280 nm and lyophilized.

Enzymatic Cleavage of the Glucoamylase Isoenzymes with Endoproteinase Lys-C

The reduced and S-carboxymethylated isoenzymes (0.25 mg) were hydrolyzed with endoproteinase Lys-C (Boehringer Mannheim, Mannheim, Germany), as described by the manufacturer, using a 1/50 enzyme/substrate ratio (w/w) in 25 m*M* Tris-HCl buffer, pH 8.5, for 16 h, at 38°C. The reactions were stopped by addition of 1% trifluoroacetic acid until pH 2.0 and the products separated by HPLC-RP C18.

Peptide Fractionation by HPLC-RP C18

The products of endoproteinase Lys C hydrolysis of the reduced glucoamylase isoenzymes were fractionated by reverse phase high performance liquid chromatography (HPLC-RP) on μBondapak C18 (3.9 × 300 mm, Waters) column. The column was previously equilibrated with 90% of eluant A (0.1% TFA) and 10% of eluant B (70% acetonitrile with 0.08% TFA). The reverse phase chromatography was carried out at room temperature at 0.5 mL/min using a Waters work station equipped with a photo diode array

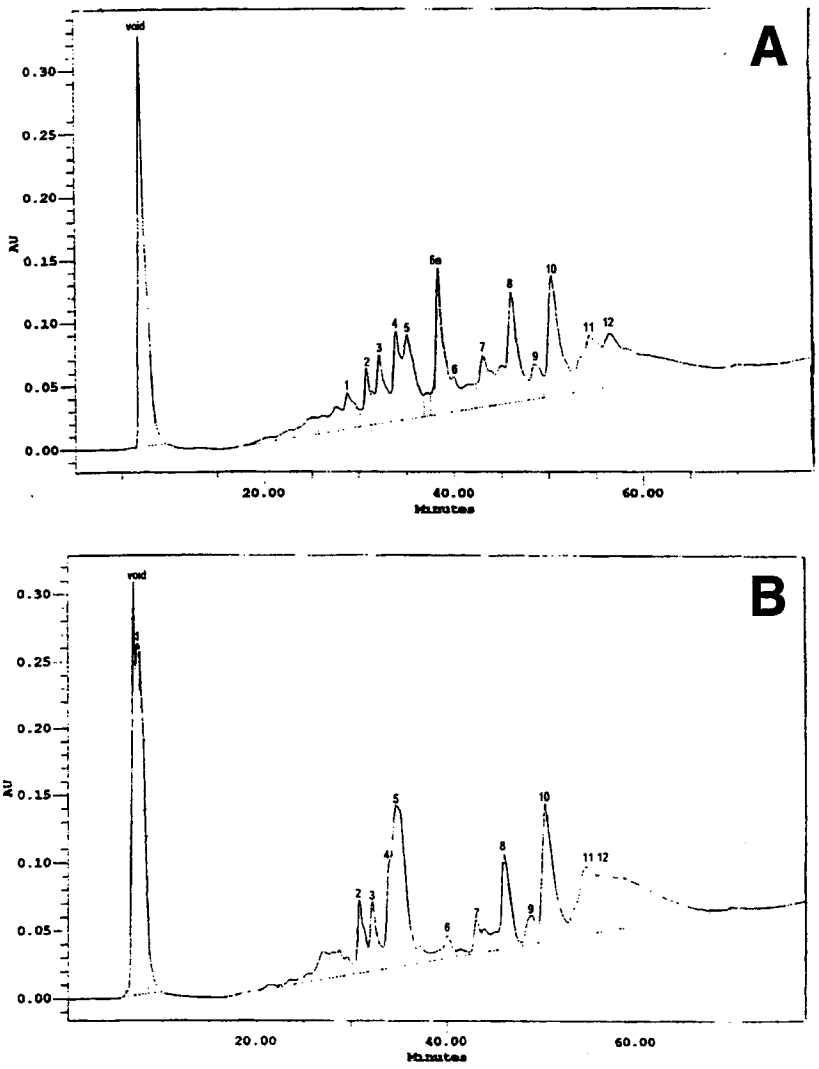

Fig. 1. Analysis of the endoproteinase Lys C hydrolysates of GAI-RCM **(A)** and GAII-RCM **(B)**. The profile corresponding to GAI-RCM hydrolysates presents a mismatched peptide 6a. Detection by absorbance at 220 nm and flow rate 30mL/h.

detector. The gradients used are indicated in the legend of Fig. 1. Samples were injected in 0.35-mL volumes and effluent was monitored at 220 and 280 nm. The relevant samples were dried in a SpeedVac system (Savant).

Purification of the Peptides Separated by Biogel P-10 Chromatography

The peptide fractions collected in the fractionating range of Biogel P-10 chromatography were fractionated by HPLC-RP C4 in a HiPore column (250 × 10 mm, Bio-rad, Richmond, CA). The reverse-phase chroma-

tography was carried out at 1.0 mL/min using a Waters work station. The elution was performed with a acetonitrile gradient in 0.08% trifluoroacetic acid (TFA) for 40 min.

Amino Acid Analysis Of Relevant Peptides

The peptides were hydrolyzed in constant boiling 5.8 *N* HCI for 22 h at 110°C under vacuum. The hydrolyzate was dried in dessicator and analyzed on a Pharmacia Biotech Biochrom 20 amino acid analyzer. The standard amino acids were from Sigma (2.5 nmoles/μL).

Analytical

Glucoamylase activity and glucose concentration determinations were performed according to previous work *(1)*. Protease activity was measured using azocasein as substrate *(8)*.

RESULTS AND DISCUSSION

Comparing the profiles corresponding to the enzymatic hydrolysis of GAI-RCM and GAII-RCM, which corresponds to the predominant isoenzyme at the end of C/N 10 and C/N 26 fermentations, respectively, it is clear that the peptide 6a from the GAI-RCM hydrolysate is absent in the GAII-RCM hydrolysate (Fig. 1). Table 1 presents a comparison between the amino acid composition of peptide 6a and its theoretical counterpart. Although the composition of the isolated peptide 6a does not precisely match the expected profile, there is a great deal of similarity between the experimental and theoretical data. The results obtained so far support previous results related to the selective production of GAI or GAII depending on the medium composition. The carbon source surplus present in the C/N 26 fermentation is a key element for the GAI transformation into GAII.

The profile of glucoamylase and protease activity during the C/N 10 and C/N 26 fermentation were determined (Fig. 2). The initial levels of glucoamylase and protease were similar in both media conditions up to fourth day. Glucose depletion occured before the fifth day of the cultivation for the C/N 10 media and, therefore, the culture died was of nutrient depletion. Considering the C/N 26 fermentation, a different pattern was observed the carbon source availability beyond this period. Although glucoamylase activity remained stable from the fourth to the seventh day, protease activity kept increasing until a peak was reached within 1 wk of fermentation. Coincidently, glucoamylase activity showed a sudden rise at this point. Considering that GAII shows a higher activity towards maltose in comparison to GAI *(1)*, this increase in enzyme activity could be caused by the generation of GAII from GAI at this point in the course of the fermentation, instead of an increase on enzyme production. Considering this possibility, the isoenzyme proportion in the culture supernatant

Table 1
Amino Acid Composition of the Peptide 6a Isolated by HPLC-Bondapak C18 from Endoproteinase Lys-C Digest of GAI-RCM

	Amino acid composition of 6a (T_R = 39 min) peptide		
Amino acid	ρ moles/μL	No. of mols/mol of peptide	Theoretical composition of peptide 6a no. of mols/mol of peptide
cysteic acid	≤2 (≤0.05)		
S-CMC	≤2 (≤0.05)		
D	60 (1.50)	1–2	1
E	126 (3.16)	3	2
S	125 (3.12)	3	3
G	85 (2.13)	2–3	1
H	4 (0.10)	0	0
R	15 (0.38)	0	0
T	120 (3.00)	3	3
A	50 (1.25)	1	1
P	75 (1.88)	2	2
Y	60 (1.50)	1–2	3
V	76 (1.90)	2	2
M	17 (0.42)	0	0
C	10 (0.25)	0	0
I	22 (0.55)	0–1	0
L	78 (1.95)	2	2
F	40 (1.00)	1	1
K	39 (0.98)	1	1
W[a]	—	—	1
TOTAL		22–25	23

[a] Residue not determined.

The amino acid composition of a theoretical peptide (Y^{554}-K^{576}) present in the C-terminal portion of GAI resulting from the same treatment is also presented.

was examined before and after the seventh day of incubation. The results that were obtained are presented in Figs. 3 and 4. There is a clear inversion of the proportion of GAI and GAII in the analyzed supernatants, suggesting the transformation of GAI into GAII after 7 d of fermentation. This transformation may be related to proteolysis as protease concentration may have reached a threshold concentration on the seventh day that allowed the occurence of glucoamylase processing. Figure 5 presents the isoenzymes profile of the C/N 10 fermentation supernatant. This profile indicates, as expected, the prevalence of GAI.

Although the foregoing evidence supports the hypothesis of extracellular processing for the generation of GAII, the definitive argument would come from the identification of the peptide Gp-1 in the culture supernatant.

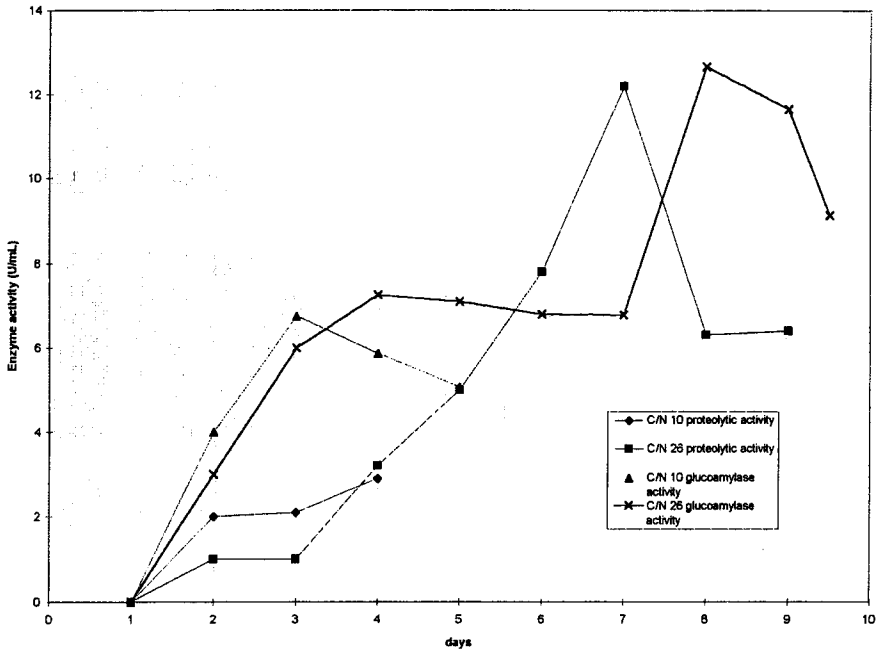

Fig. 2. Profile for glucoamylase and protease activity in C/N 10 and C/N 26 fermentations.

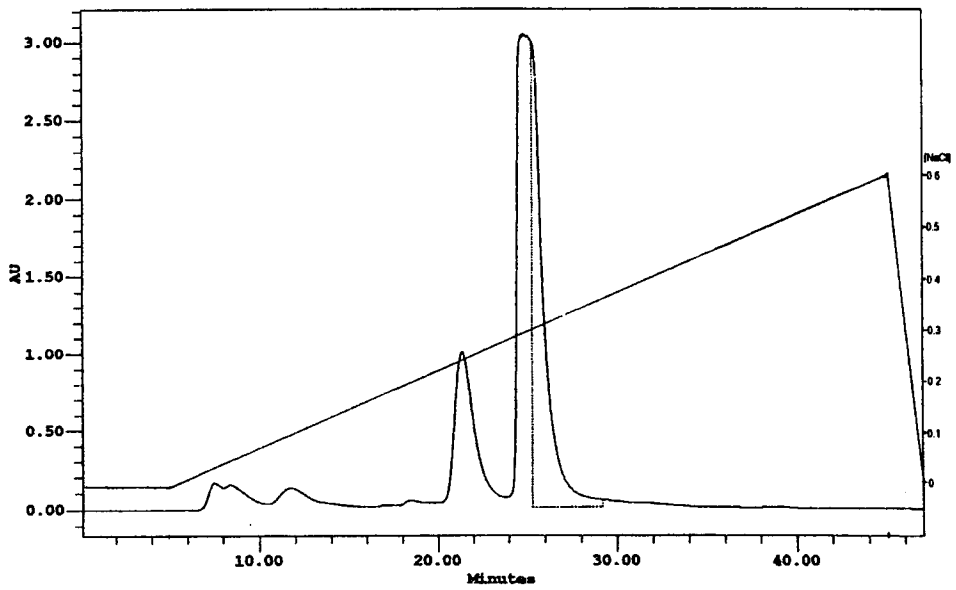

Fig. 3. Ion-exchange chromatography in FPLC-mono Q column of the culture supernatants and GAI from 3 and 4 d of C/N 26 fermentation, showing GAII (peak with retention time 21.5 min) and GAI (peak with retention time of 25.2 min).

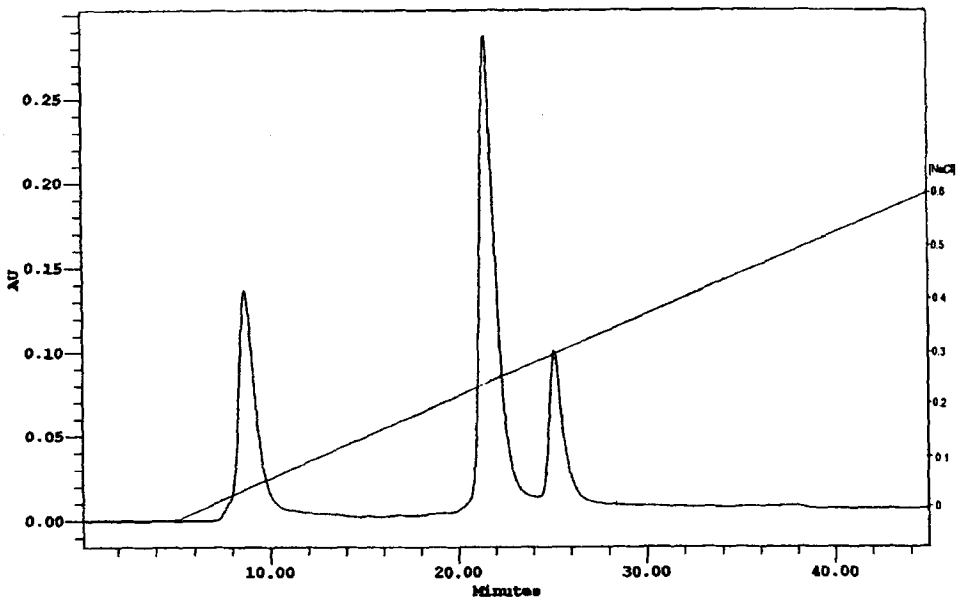

Fig. 4. Ion-exchange chromatography in FPLC-mono Q column of the culture supernatants and GAI from 8 and 9 d C/N 26 fermentation, showing GAII (peak with retention time 21.5 min) and GAI (peak with retention time of 25.2 min).

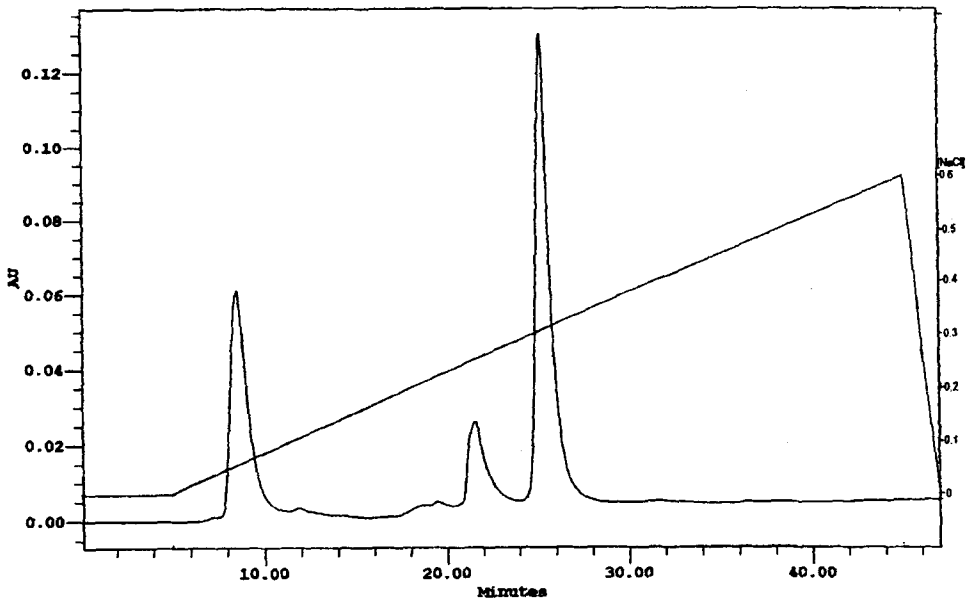

Fig. 5. Ion-exchange chromatography in FPLC-mono Q column of the culture supernatants and GAI from 3 and 4 d of C/N 10 fermentation, showing GAII (peak with retention time 21.5 min) and GAI (peak with retention time of 25.2 min).

Table 2
Retention Time (R_T) of HPLC-RP C4 for Peptide I and Peptide II Fractions Isolated from Biogel P-10 Column

	C/N 26 medium	
Day	Peptide I	Peptide II
4	31 min[a], 38 min, 34 min, and 39 min	24 min, 26 min, 30 min 34 min, and 36 min
7	31 min,[a], 38 min and 39 min	26 min,[a] 30–39 min
8	17.5 min,[a] 38 min, and 39 min[a]	20 min[a] and 26 min[a]
9	18.5 min, 19 min,[a] 38.5 min, and 36.5 min	18 min and 18.5 min[a], 19 min and 19.5 min

[a] The major peptide at 220 and 280 nm detection.

Obs.: the peptide II R_T = 26 min related to 7 and 8 d fermentation of C/N 26 fermentation was isolated and had its amino acid composition determined.

Accordingly, Bio-gel P10 chromatography was used in this study to separate the glucoamylase proteins, which eluted in the void volume, and to fractionate peptides with molecular weight within 1500 and 20,000. This methodology could resolve Gp-1, as it shows a molecular weight of 11,000 (100 amino acid residues). Two peptide fractions were resolved from the supernatant of the C/N 26 fermentation mainless of the fermentation time. These fractions, however, showed different V_e/V_o values. The fractions corresponding to the fourth day showed the value of 1.34 (peptide 1) and the value of 1.40 (peptide 2). Peptides 1 and 2 from the seventh eighth, and ninth days presented the same V_{eo} values, i.e., 1.67 and 1.70, respectively. The HPLC-RP-C4 profiles from peptide 1 and peptide 2 showed at fourth and seventh days peptide fractions with high retention times, whereas the eighth and ninth days samplings resulted in fractions with low retention times, suggesting a continuous degradation (Table 2). The main peptide fraction (retention time 26 min) from HPLC-RP C4 profiles of peptide 2 from seventh and eighth day, was collected and subjected to amino acid composition analysis (Table 3). This amino acid composition was similar to a theoretical peptide produced by the cleavage in the residues G^{526} and A^{551} from the C-terminal portion of GAI.

CONCLUSIONS

Structural analysis of GAI and GAII glucoamylase isoenzymes confirmed the effect of the medium C/N ratio on the selective production of GAI or GAII in submerged fermentations of *Aspergillus awamori*. Carbon limited fermentations favors GAI predominance, whereas nitrogen limitation favors GAII formation. The *in vivo* extracellular mechanism responsible for the formation of GAII seems to involve proteolytic processing, because the conversion of GAI into GAII during the fermentation was

Table 3
Amino Acid Composition of a Reverse-Phase Peptide (R_T = 26 min) Isolated from Peptide II Fraction HPLC-RP C4a 7 and 8 d

Amino acid	ρ noles	Σρ noles	Assumed D+E = 6.00	GA_I theoretical peptide (G^{526}-A^{551})
D+E	380+860	1240	6.00 (6)	6
T+S+G	270[a]+635[a]+960	1865	9.02 (9)	9
A+V+I+L	440+400+250+5 70	1660	8.03 (8)	8
Y+F	60+150	210	1.02 (1)	1
P	≤50	≤50	≤25 (0)	0
C	≤50	≤50	≤25 (0)	0
M	≤50	≤50	≤25 (0)	0
R	≤50	≤50	≤25 (0)	0
			24	24

[a] Values corrected considering 8% for T destruction and 15% for S destruction.

An amino acid composition of a theoretical peptide (G^{526}-A^{551}) present in the C-terminal portion of GAI is also showed. The amino acids were grouped according to its similarities. The lysine and histidine residues were lost.

concomitant with high proteolytic activity in the culture medium. The medium C/N ratio also effects protease production that is favored in nitrogen-limited fermentations. The presence of peptide Gp-1 was not identified in the culture supernatant. It was possible, however, to identify a smaller peptide that may have resulted from the continuous degradation of the C-terminal peptide Gp-1. This peptide matched with the region G^{14}-A^{39} of Gp-1, according to the hypothesis of the extracellular proteolytic processing of GAI.

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