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Glucoamylase Isoenzymes Tailoring Through Medium Composition

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ABSTRACT

Two major glucoamylase isoenzymes (GAI and GAII) have been identified in culture supernatants of *Aspergillus awamori*. It has been suggested that a stepwise degradation of a native enzyme during the fermentation by proteases and/or glucosidases results in the formation of isoenzymes that have different characteristics concerning substrate specificity and stability to pH and temperature. In this study, the glucoamylase isoenzymes produced by *Aspergillus awamori* using liquid media with C/N 10 (2.0% starch, 0.45% $(NH_4)_2 SO_4$) and C/N 26 (5.2% starch, 0.45% $(NH_4)_2 SO_4$) were analyzed. In both cases, GAI and GAII were characterized concerning its hydrolitic activities, mol wt, and isoeletric point. Using HPLC gel filtration and FPLC chromatofocusing, it was obtained for GAI a mol wt of 110,000 Da, pI 3.45 and for GAII a mol wt of 86,000 Da, pI 3.65. A different isoenzymes proportion was observed by the use of the two C/N ratios. In the lower carbohydrate content, fermentation of the GAI form predominated, whereas in the C/N 26 medium, GAII was prevalent. Gel eletrophoresis, amino acid analysis, and structural data confirmed that both preparations were glucoamylases with a high degree of homogeneity.

Index Entries: *Aspergillus awamori*; glucoamylase production; glucoamylase characterization; isoenzymes proportion; medium C/N ratio.

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INTRODUCTION

Amylolytic enzymes from filamentous fungi have been extensively investigated because of their importance in the starch industry *(1)*. Glucoamylase [α-*(1–4)* glucan glucohydrolase, [E.C.3.2.1.3] was identified in the early 1950s, and the existence of two isoenzymes produced by *Aspergillus niger* was firstly reported in 1959 *(1,2)*. Since then the presence of two isoforms, glucoamylase I and II (GAI and GAII), have been consistently identified in fermentations carried out using several genera and species of fungi *(3–12)*. Some authors, however, have also reported the existence of a higher number of glucoamylase isoenzymes that could be related to variable glycosylation patterns *(13–20)*.

A great deal of work has been carried out aimed at the characterization of the two major isoenzymes, GAI and GAII concerning their physicochemical and catalytic properties *(11,12,21,22)*. Both forms consist of a single glycosylated polypeptide chain with typical mol wt of 75,000 Da and 54,000 Da, respectively *(7)*, although the molecular weights of these glycoproteins vary according to the analytical procedure used *(21)*. The polypeptide chain of the two forms are very similar, however GAI and GAII have different C-terminal residues. GAII differs from GAI by lacking a COOH-terminal region of approx 100 amino acids residues *(23)*. The isoforms can be distinguished in their function, the GAI (614–616 amino acids) having substantial activity toward raw starch in contrast to GAII (512 amino acids). This difference is atributted to the absence in the GAII molecule of a C-terminal peptide (glycopeptide Gp-1) that corresponds to a raw starch-binding domain *(21,24–27)*. It is reported that the isoenzymes show the same activity towards soluble substrates *(23)*.

The mechanism involved in the generation of the isoenzymes is debatable. Molecular cloning and characterization of the glucoamylase gene of *Aspergillus awamori* demonstrated that the glucoamylase gene exists as a single copy gene *(28)*. Thus the mechanisms for the generation of the isoenzymes would be related to either differential mRNA splicing or posttranslational modifications. Two different glucoamylase cDNA were identified, a major form coding for GAI and a minor, smaller one, originating from an additional mRNA splicing within sequences encoding the C-terminal position of GAI *(28,29)*. However, since the structure of the smaller form differed from its presumptive mRNA in the C-terminal sequence, it was suggested that GAII was generated by posttranslational proteolysis *(30)*. This possibility was later addressed. The limited in vitro degradation of GAI using fungal acid proteases or subtilisin resulted in the production, by liberating the glycopeptide GpI, of a GAI' that was a raw starch nondigesting glucoamylase *(31–33)*. These results were later confirmed by the determination of the amino acid sequence of both N- and C-terminal of Gp-I obtained by subtilisin cleavage of GAI and its insertion on the complete amino acid sequence of GAI *(18)*.

The effect of the growth medium composition and the presence of extracellular proteases on the proportion of the isoenzymes was studied. A relationship was observed between proteolytic activity and the conversion of GAI into GAII during the course of the fermentation. Longer fermentations also favored the conversion process *(15,34)*. Moreover, only one type of glucoamylase, GO-0, (raw starch digestive) was obtained from a protease-negative, glucosidase negative *Aspergillus awamori* mutant *(33)*.

The main aim of the present work was to investigate the effect of the growth medium C/N ratio on the glucoamylase isoenzymes produced by *Aspergillus awamori* using ammonium sulfate as a nitrogen source and starch as a carbon source. Growth medium limited by carbon or nitrogen were designed by keeping the nitrogen source concentration constant and using a low and a high carbon source concentration *(35)*. Under these conditions, shorter and longer fermentations were observed. The profile of glucoamylase isoenzymes from both conditions were compared. A higher proportion of GAI was observed under carbon limiting conditions while GAII prevailed on the nitrogen limited medium. These data suggest that the short fermentation condition diminished the processing of GAI by proteases in the culture medium, most probably because of a lower degree of cell lysis that dimished the enzyme liberation into the media. The repression of protease production in such an ammonium rich medium is also a possibility to be explored *(36)*.

MATERIAL AND METHODS

Culture Maintenance and Propagation

All procedures were carried out according to previous work where the same strain, *Aspergillus awamori* 2.B.361 U2/1 was used *(35,37)*.

Fermentations

Shaken flasks fermentations were carried out using growth medium containing 0.45% ammonium sulphate and 0.1% yeast extract plus starch 1.9% (medium A, C/N ratio 10) or 5.2% (medium B, C/N ratio 26). Medium A and B were carbon and nitrogen limited, respectively, as a balanced medium using ammonium sulfate as nitrogen source shows a C/N ratio of 15 *(35)*. Shaken flasks containing 200 mL of the growth medium were incubated in a rotatory shaker at 30°C and 250 rpm. After glucose consumption, the mycelium was separated from the culture medium through filtration using a Buchner funnel and glass microfiber filter (Whatman GF/A). The culture supernatant was collected for further analysis.

Crude Glucoamylase Isolation

The culture supernatants (around 80 mL) from growth media A and B were concentrated by liophylization to half of its initial volume and chromatographed in a Biogel P-6 colunm (50 × 5 cm, eluant 0.1*M* acetic

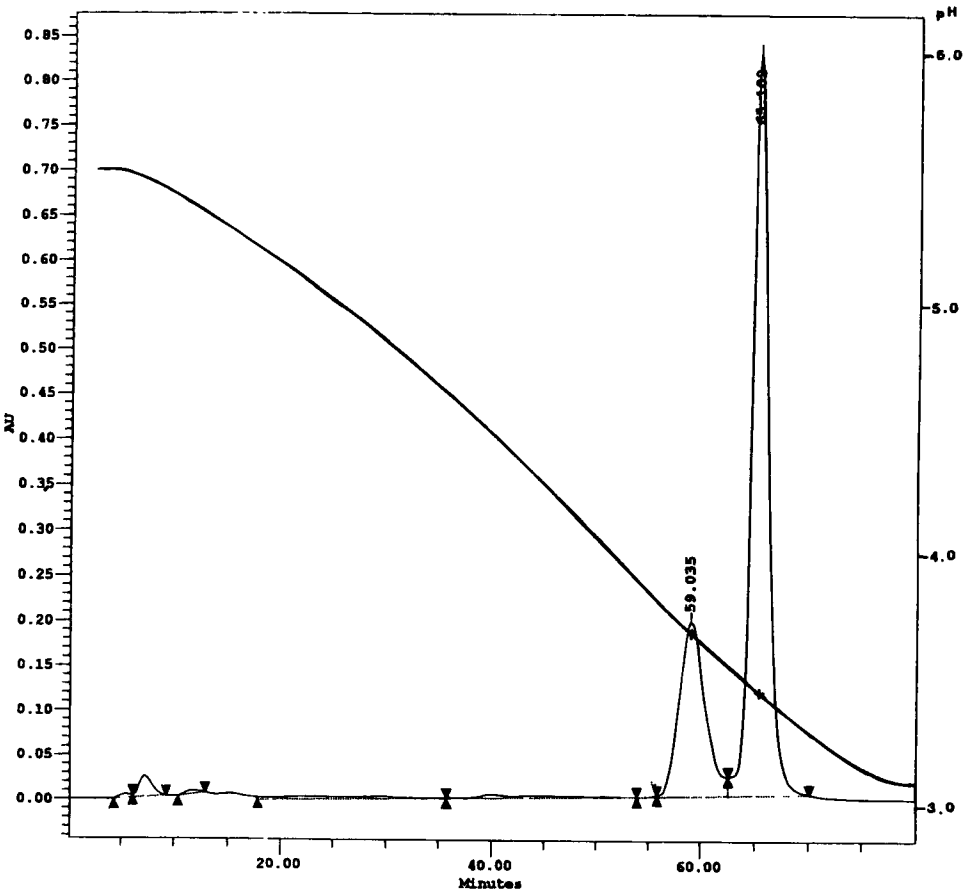

Fig. 1. FPLC-Chromatofocusing of 3.0 mg of crude glucoamylase preparation originating from medium A (C/N 10). Detection at 280 nm: Form I (pI = 3.45) and Form II (pI = 3.65).

acid and flow rate 35 mL/h) for the separation of the protein fraction from low-mol-wt compounds (MW < 6000 Da). The effluent was continuously monitored at 280 nm and the sole protein peak eluted in the column void volume, for medium A and B (crude glucoamylases), was collected and lyophilized.

Chromatofocusing of the Crude Glucoamylase Preparations

The two crude glucoamylase preparations were submitted to chromatofocusing in a mono P column (HR 5/20). The column was equilibrated with L-His 25 m*M* pH 5.5 and the pH gradient was established by the addition of polybuffer 74 (1/15, v/v in water), pH 3.1. The sample (3.0 mg dissolved in the start buffer, pH 5.5) fractionation was performed during 5 min in initial conditions followed by 85 min with the polybuffer solution, using a flow rate of 45 mL/h. The column efluent was used for pH

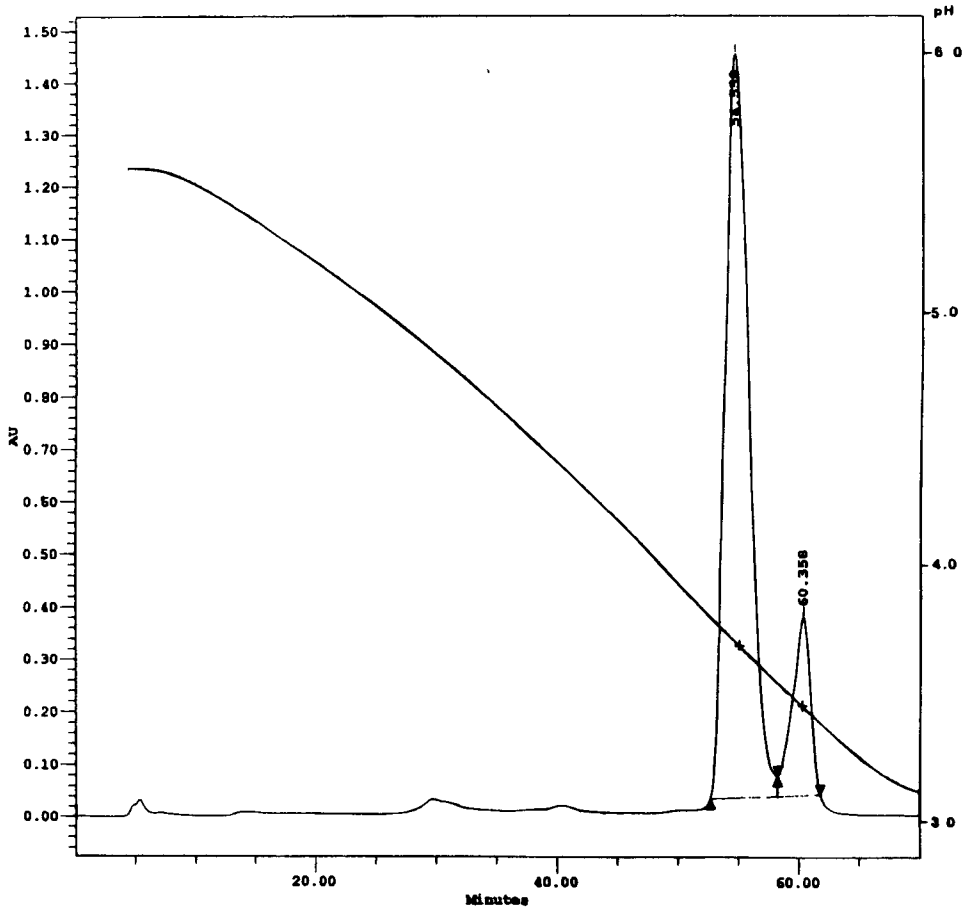

Fig. 2. FPLC-Chromatofocusing of 3.0 mg of crude glucoamylase preparation originating from medium B (C/N 26). Detection at 280 nm: Form I (pI = 3.45) and Form II (pI = 3.65).

and protein concentration determination (absorbance at 280 nm). Two main fractions, Form I, pI 3.45 (Fig. 1, retention time 65.102 min; Fig. 2, retention time 60.358 min) and Form II, pI 3.65 (Fig. 1, retention time 59.035 min; Fig. 2, retention time 54.558 min) were observed in the two crude glucoamylase preparations. Form I predominated in the preparation originating from medium A and Form II was the major component in the preparation from medium B. The fractions were concentrated in a speed vac and desalted in Biogel P-10. Both fractions from both growth media presented glucoamylase activity.

Native and Denaturing Polyacrylamide Gel Electrophoresis

The two crude glucoamylase preparations were submitted to native and denaturing discontinuous polyacrilamide gel electrophoresis (8–25% gradient gel) in a Phast System of Pharmacia using mol wt standards

within the range of 20,000 to 200,000 Da for the calibration curve. In the denaturing gel electrophoresis the samples were previously incubated with SDS and dithiothreitol at 100°C for 5 min in 0.1*M* Tris-HCl buffer, pH 6.8. In all cases the gels were stained with CBR-250 and scanned in a LKB laser densitometer.

HPLC-Gel Permeation

The crude glucoamylases and Form I and Form II preparations were chromatographed in protein pak 300 SW (Waters). The column (30.0 × 7.8 mm) elution (30 mL/h) was performed using 0.2*M* phosphate buffer pH 6.0 and the effluent was monitored at 280 nm. Molecular weight standards within the range of 20,000 to 150,000 Da were used for the calibration curve.

Amino Acid Analysis

Form I chromatofocusing preparation from medium A and Form II from medium B were hydrolyzed in 5.8 N HCl vapor phase at 110°C for 22 h. The dried hydrolysate was derivatized with PITC, according to manufacturer, and analyzed in a PICO-TAG column (Waters). Each analysis was performed in duplicate.

Amino Acid Sequence

Glucoamylases Form I and II were submitted to *N*-terminal amino acid sequencing in a Shimadsu Sequenator. Approx 300 pmoles of the Form I and 250 pmoles of Form II were loaded into the sequenator.

Analytical

Protein concentration was measured using the SDS Folin-Lowry method. Enzyme and glucose concentrations were performed according to previous work *(35,37)*. The carbohydrate content of crude glucoamylases from medium A and B was determined using the phenol sulfuric method *(38)*.

RESULTS

Fermentations using medium A finished within 4 d whereas when medium B was used, glucose depletion ocurred within 10 d. A higher glucoamylase activity was observed in the culture supernatant of medium B, suggesting the presence of a more active molecule (Table 1). Medium B supernatant also presented a higher protein content: 261 mg of protein were recovered after Biogel P-6 chromatography in comparison to 40.6 mg from medium A. SDS-PAGE experiments showed one major peak (mol wt 110,000 Da) and three minor peaks, poorly resolved (mol wt 62,000; 65,000, and 70,000 Da) for medium A glucoamylase. An opposite pattern was observed for medium B glucoamylase, i.e., a minor component (mol wt 100,000 Da) and a protein family showing mol wt within 60,000–70,000 Da.

Table 1
Glucoamylase Activity of the Culture Supernants and Form I and II using Maltose (a) and Starch (b) as Substrate[a]

Glucoamylase activity ($U.mg^{-1}$)					
Culture Supernatants		Chromatofocusing Preparation			
Medium A (C/N 10)	Medium B (C/N 26)	Medium A (C/N 10)		Medium B (C/N 26)	
		Form I	Form II	Form I	FormII
$4.0^{(a)}$	$10.0^{(a)}$	$2.0^{(a)}$	$1.8^{(a)}$	$4.2^{(a)}$	$8.4^{(a)}$
		$18.7^{(b)}$	$13.1^{(b)}$	$23.6^{(b)}$	$64.0^{(b)}$

[a]Enzyme activity was expressed as μmol of glucose produced per minute per mg of protein ($U.mg^{-1}$) under inital rate conditions at 40°C.

Two major fractions with pH values 3.45 and 3.65 were observed after the chromatofocusing analysis of the crude glucoamylases. In preparation from medium A, the fraction with pI 3.45 (Form I) prevailed (80%) whereas the fraction with pI 3.65 (Form II) was predominant in preparations from medium B (85%), as shown in Figs. 1 and 2. The Form I and the Form II chromatographed by HPLC protein pak 300SW showed one peak with different retention times whose mol wt were 106,000 and 85,000 Da.

The amino acid composition of Form I and Form II (Table 2) relates closely to the reported composition of GAI and GAII *(22,23)*. The partial *N*-terminal amino acid sequence of the two isoenzymes isolated by chromatofocusing gave "clean" sequence until the fifteenth amino acid residue, which was consistent with the sequence known for GAI and GAII *(22,23)*.

According to the foregoing of the physicochemical characterization of the polypeptide chain of the glucoamylase preparations allowed, the identification of the two major isoenzymes, in different proportions, in the C and N nitrogen-limited medium. The isoforms activities varied according to the medium C/N ratio (Table 1). Isoenzymes from medium A (C/N ratio 10*)* presented equivalent activities toward maltose and soluble starch, whereas Form I and Form II from medium B (C/N ratio 26) were, respectively, two and four times more active in maltose in comparison to its medium A counterparts. Form II was, at least, three times more active in starch than all other isoforms.

The determined carbohydrate content of crude preparations from medium A and B were of 13.7 and 34.2%, respectively. Therefore, a higher degree of glycosylation occured in the isoforms from medium B that presented a higher carbohydrate surplus. Coincidently, crude glucoamylase B (medium C/N ratio 26) presented a carbohydrate content 2.5 times higher

Table 2
Amino Acid Composition (PICOTAG column, HCl 5.8N vapor phase hydrolysis) of Glucoamylases Preparations Form I and II, Isolated by FPLC-Chromatofocusing in mono P Column[a]

Amino acids	pmoles/μl (Form I)	pmoles/μl (Form II)	Reported compositions (GAI)[22]	Reported composition (GAII)[23]
D	95.34 (64)	72,15 (55)	66	56
E	59.57 (40)	40,89 (31)	41	31
S	107.43 (85)*	71,00 (63)*	87	76
G	68.09 (46)	47,70 (36)	45	40
H	7.40 (05)	5,54 (04)	04	04
R	27.48 (18/19)	19,51 (15)	18	15
T	91.41 (69)	61,63 (52)*	73	58
A	88.96 (60)	68,28 (51/52)	63	53
P	35.40 (24)	24,70 (19)	22	19
Y	40.90 (28)	25,08 (19)	27	21
V	53.98 (36)	44,05 (33)	41	33
M	4.85 (03)	4,31 (03)	02	02
C	10.46 (09)	6,41 (06)*	09	08
I	31.19 (21)	21,93 (16/17)	23	18
L	55.72 (37/38)	43,88 (33)	43	37
F	29.69 (20)	22,53 (17)	20	17
K	18.86 (13)	13,49 (10)	12	10
W	———	———	18	14
Number of residues	577-580	462-465	614	512

*Correction for acid destruction: 15% for serine (S), 10% for threonine (T), and 20% for cysteine (C).

Note: tryptophane (W) were not determined.

[a]In parenthesis are shown the number of amino acid residues/mol. The data are average values for two analyses of the same hydrolyzate.

in comparation to crude glucoamylase A (medium C/N ratio 10) and, therefore, protein glycosylation responded proportionally to the increase in the medium C/N ratio. As previously stated, the isoforms from medium B presented higher activity.

The similar patterns of activities toward maltose and starch presented by Form I and II from medium A, indicated that the lack of the raw starch binding domain did not have a major effect on the enzyme activity towards soluble substrates.

DISCUSSION

Structural and catalytic data allows the conclusion that the glucoamylases preparations, Form I and II, which were obtained by chromatofocusing, corresponds to the GAI and GAII isoenzymes molecules. The relative

proportion of the isoenzymes were different prevailing GAI in medium A (C/N ratio 10) and GAII in medium B (C/N ratio 26). In this case, GAII showed to be two times more active in relation to GAI in maltose substrate and three times higher in starch (Table 1). The data concerning the eletrophoretic and chromatographic experiments are consistent with the hypothesis that extacellular proteases processes GAI, transforming it into GAII. This assumption is supported by the isoenzymes (Form I and II) amino acid compositions. Intracellular processing proteases are known to attack peptides linkages in dibasic sites. This is not the case for GAI transformation into GAII, as the C-terminal amino acid in GAII is alanine. Therefore, the GAI processing would involve an extracellular protease. The identification of the peptide Gp-1 in the extracellular environment would be a definitive argument for extracellular protease processing. In conclusion, media composition affected the isoenzymes proportion in the culture supernatant. The characterization of this effect can be used to obtain glucoamylases with particular characteristics. This possibility could be further explored as the presence of isoenzymes is a common pattern in extracellular fungi enzymes.

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