Evidence that the glucoamylases and α-amylase secreted by *Aspergillus niger* are proteolytically processed products of a precursor enzyme

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Abstract A 125-kDa starch hydrolysing enzyme of *Aspergillus niger* characterised by its ability to dextrinise and saccharify starch [Suresh et al. (1999) Appl. Microbiol. Biotechnol. 51, 673–675] was also found to possess activity towards raw starch. Segregation of these activities in the 71-kDa glucoamylase and a 53-kDa α-amylase-like enzyme supported by antibody cross-reactivity studies and the isolation of mutants based on assay screens for the secretion of particular enzyme forms revealed the 125-kDa starch hydrolysing enzyme as their precursor. N-terminal sequence analysis further revealed that the 71-kDa glucoamylase was the N-terminal product of the precursor enzyme. Immunological cross reactivity of the 53-kDa amylase with antibodies raised against the precursor enzyme but not with the 71- and 61-kDa glucoamylase antibodies suggested that this enzyme activity is represented by the C-terminal fragment of the precursor. The N-terminal sequence of the 53-kDa protein showed similarity to the reported Taka amylase of *Aspergillus oryzae*. Antibody cross-reactivity to a 10-kDa non-enzymic peptide and a 61-kDa glucoamylase described these proteins as products of the 71-kDa glucoamylase. Identification of only the precursor starch hydrolysing enzyme in the protein extracts of fungal protoplasts suggested proteolytic processing in the cellular periplasmic space as the cause for the secretion of multiple forms of amylases by *A. niger*.

Key words: Glucoamylase; α-Amylase; Amylase precursor; Raw starch hydrolysis; *Aspergillus niger*

1. Introduction

An interesting feature of *Aspergillus niger* is the number of amylases characterised from strains of this fungus [1]. Though the variations in the molecular masses of the two glucoamylases G1 and G2 secreted by the fungus have been suggested to be due to differential splicing of the intervening sequences in the mRNA [2], the occurrence of the two forms of the enzyme was suggested to be due to post-translational modifications of the protein [3].

In culture, *A. niger* was reported to secrete an α-amylase [4] and glucoamylases of varying molecular masses (reviewed in [5]). While glucoamylases possess inherent saccharifying α-1,6 and α-1,4 exo-activity to starch and related maltodextrins [6] and are in most cases the major enzymes, the need for the secretion of other amylases by the fungus is not entirely clear. Recently, a starch hydrolysing enzyme secreted by *A. niger* was characterised [7]. In this paper, evidence is presented for a proteolytic processing of the starch hydrolyzing enzyme which results in the secretion of glucoamylases and α-amylase by the fungus.

2. Materials and methods

2.1. Immunological methods

Antibodies directed against the 125-kDa starch hydrolysing enzyme [7] and the 71- and 61-kDa glucoamylases (purified from the culture broth of *A. niger* [8]) were raised in rabbits by injecting 13 μg of protein [9] via the intramuscular route with Freund's complete adjuvant on days 0, 14, 28 and 42 [10]. Antisera obtained by marginal ear vein bleeding were used for Western blot analysis of proteins separated on 10% SDS–polyacrylamide gels [11] and electroblotted onto nitrocellulose membranes [12]. After blocking the membranes in 15% skimmed milk solution, the proteins were identified using the antibody (1:200 dilution) and anti-rabbit goat IgG tagged with peroxidase (1:400 dilution in 10 mM Tris–HCl buffer saline, pH 8.0; Sigma). Hydrogen peroxide (0.025%) and 4-chloro-1-naphthol (0.04%) in 50 mM Tris–HCl buffer (pH 7.6) were used as substrates for the enzyme reaction. In some of the experiments, protein extracts obtained from the fungal protoplasts [13] were used. The proteins, separated by SDS–PAGE and Western blots, were developed as described above. Affinity purification of proteins present in the crude culture filtrates was carried out using antibody immobilised on nitrocellulose membranes [14]. The eluted proteins were identified by silver staining [15] after electrophoretic separation on 10% SDS–polyacrylamide gels.

2.2. N-terminal sequencing

The purified proteins were separated on 10% SDS–PAGE gels and electrophoretically transferred to PVDF (polyvinylidene difluoride) membranes using 0.1 M CAPS [3-(cyclohexylamino)-1-propane-sulphonic acid] buffer, pH 11.0. Microsequencing was performed using the membrane-bound protein and sequence identities were detected by a BLAST search at NCBI.

2.3. Enzyme assay

An amylase assay was carried out at 37°C using 1% Lintner's soluble starch (BDH Laboratories, Poole, UK) prepared in 0.1 M sodium acetate buffer (pH 4.3). After 60 min of incubation, the total reducing sugars and glucose released were measured using the 3,5-dinitrosalicylic acid reagent [16] and the glucose oxidase–peroxidase reagent [17], respectively.

Raw starch hydrolysing activity was identified by enzyme zymograms on isoelectric focusing gels [8]. Reactivity of the enzyme towards raw granular corn starch was also confirmed by scanning electron microscopic studies [18]. For this, 15% raw granular starch suspended in 0.1 M sodium acetate buffer (pH 4.3) was incubated with the enzyme at 37°C (10 μg enzyme protein g^{-1} substrate) for different periods of time. The samples were dehydrated in ethanol, lyophilised and observed under a Leo 435VP scanning electron microscope (SEM) after gold coating using the Polaron SEM coating system. Untreated starch granules were used as a control. Raw starch hydrolysis was simultaneously assayed by estimating glucose in the product as stated above.

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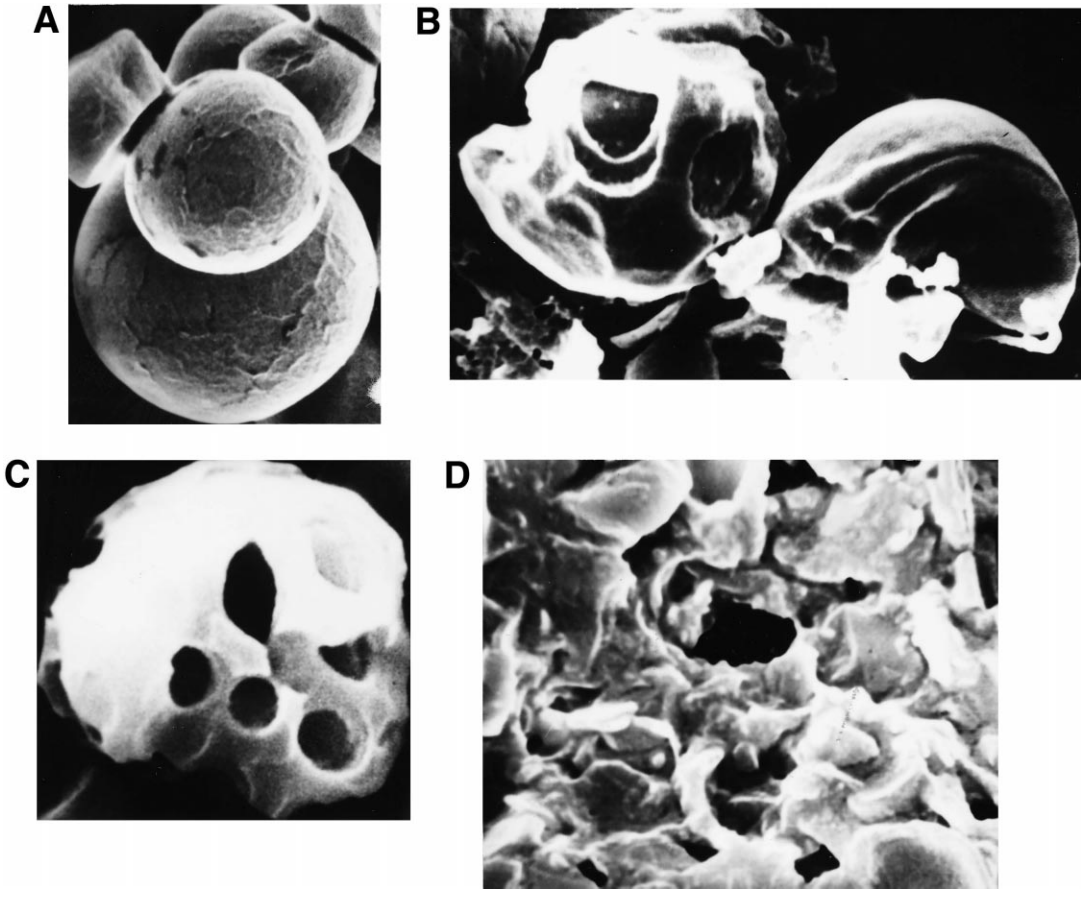

Fig. 1. Scanning electron micrographs of raw corn starch granules showing (B) facial fracture, (C) multiple pitting and (D) flaking due to activity of the 125-kDa amylase. A: Untreated starch granules (control).

2.4. Isolation of mutants

Dextrinising and saccharifying 125-kDa amylase and 71-kDa glucoamylase overproducing mutants of *A. niger* were isolated as follows: a young spore suspension of the parent culture (*A. niger*, CFTRI 1105) was plated on a starch–agar medium containing infusion from 200 g boiled potatoes, 1% starch and 2% agar (pH 5.0). The plates were inverted on a transilluminator (302 nm) and mutation was carried out to obtain a 90% kill [19]. The plates were incubated at 4°C overnight in the dark and colonies emerging after incubation at 30°C for 24 h were transferred onto fresh starch–agar plates. Presumptive mutant colonies showing large clearance zones due to starch hydrolysis were identified after flooding the plates with iodine solution (1.25% KI and 0.125% I_2) and screened for the production of enzymes of interest by growing the cultures in starch broth (4% corn flour, 0.5% corn steep liquor; pH 5.0). Qualitative assays were carried out with culture filtrates after 48 h growth. The 71-kDa glucoamylase overproducing mutant (CFTRI 1105-43) was selected based on maximum glucose release. Rapid clarification of 2% gelatinised granular corn starch in a 10 min assay reaction identified by starch iodine reaction was used to select mutant (CFTRI 1105-41) overproducing 125-kDa amylase. The culture filtrates of the selected mutants were further analysed by SDS–PAGE to confirm overproduction of the respective enzymes.

3. Results and discussion

Recently, we described the characterisation of a 125-kDa starch hydrolysing enzyme secreted as a major protein by a mutant strain of *A. niger*. The main feature of this glycosylated protein (containing ~25% mannosyl residues) of p*I* 4.0 was its ability to dextrinise and saccharify starch [7]. The zymograms of the enzyme after isoelectric focusing identified a raw starch hydrolysing activity and its adsorption to raw granular starch causing facial fracture that progressed to multiple pitting and flaking of the granules (Fig. 1) resulting in the release of 40–45% glucose within 3 days. Further hydrolysis of the starch granules was not evident despite further incubation for more than 10 days. This could not be attributed to loss of the enzyme function because activity could be assayed when enzyme-containing starch flakes were incubated with gelatinised starch at 37°C.

A peculiar feature of the antibodies raised against the 125-kDa enzyme protein was their reactivity towards proteins with molecular masses of 71, 61 and 53 kDa and co-purification of these proteins using an antibody affinity chromatography (Fig. 2). To rule out the possible presence of some of these

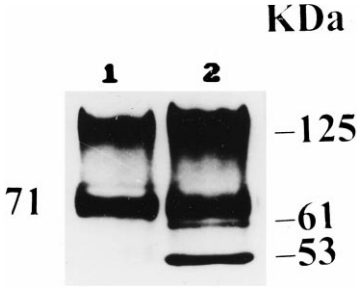

Fig. 2. Proteins of *A. niger* crude culture filtrate purified by immunoaffinity using nitrocellulose membrane-immobilised antibodies raised against [1] 71- and 61-kDa glucoamylases and [2] the 125-kDa precursor enzyme. The proteins were identified by silver staining after separation on 10% SDS–PAGE gels.

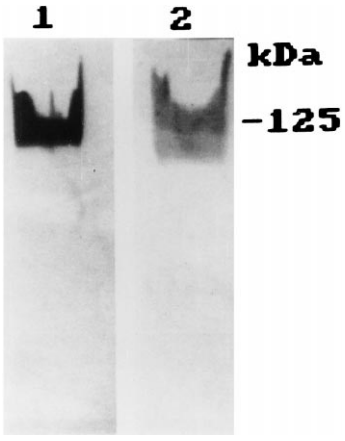

Fig. 3. SDS–PAGE of silver stained 125-kDa enzyme (15 μg pure protein) of *A. niger* [1] and corresponding Western blots [2].

proteins as contaminants in the enzyme preparation used for immunisation, we re-assessed the purity of enzyme preparation by silver and antibody staining of overloaded protein separated on 10% SDS–PAGE gels. The absence of any contaminating proteins (Fig. 3) confirmed the antibody cross-reactivity described above. Assays with gelatinised starch using each of the proteins separated on a Bio-Gel P-100 column showed a typical exo-activity characteristic of glucoamylases of the 71- and 61-kDa proteins and a dextrinising activity leading to the production of ~50% dextrose equivalent (DE) of the 53-kDa α-amylase (Fig. 4). While raw granular starch hydrolysing activity could not be identified in the 61- and 53-kDa enzymes [8], the activity on adsorption of 71-kDa glucoamylase onto raw starch resulted in the maximum release of only 15% glucose which was attributed to pit formation alone (Fig. 5). The 71-kDa glucoamylase could be differentiated from the 125-kDa amylase by different isoelectric points (p*I* 3.8 and 4.0, respectively) observed in enzyme zymograms separated on isoelectric focusing gels (Fig. 6). Since the activities described for the 125-kDa amylase appeared to segregate into the 71- and 53-kDa enzymes, antibody cross-reactivity suggested that proteolytic processing of the 125-kDa precursor form resulted in the generation of the 71- and 53-kDa amylases. This conclusion was also supported by the reactivity of antibodies raised against the 71- and 61-kDa enzymes towards the 125-kDa precursor but not towards the 53-kDa α-amylase (Fig. 2).

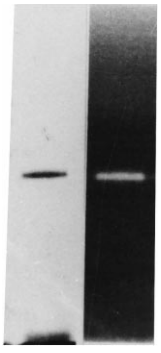

Fig. 4. Dextrinising activity of the 53-kDa protein identified by enzyme zymogram (right) after separation of the pure protein (silver stained; left) in 1% Lintner's soluble starch incorporated 10% SDS–PAGE. The gels were washed in 0.1 M acetate buffer (pH 4.3) and incubated in the buffer for 10 min. The zymogram was developed by staining the gels with iodine solution (1.25% KI and 0.125% I_2).

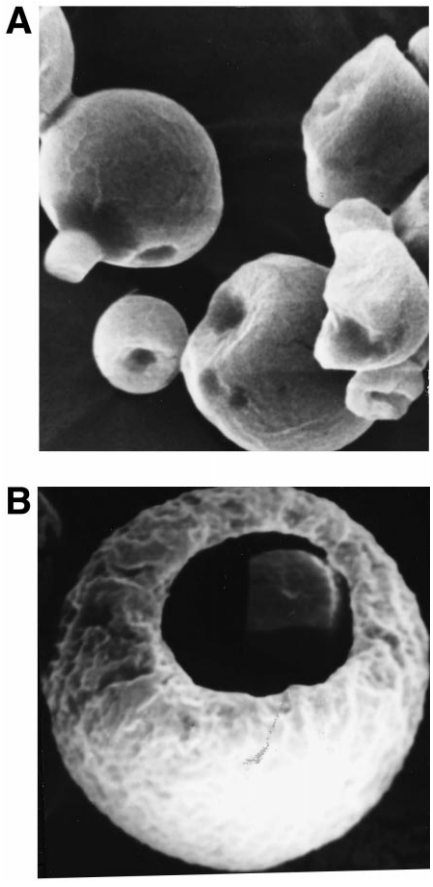

Fig. 5. Scanning electron micrographs of raw granular starch reacted upon by the 71-kDa glucoamylase showing pit formation (A). A single pitted granule (enlarged, B).

Facial fracture of raw starch granules is a typical feature of α-amylase activity and Ueda described the need for an α-amylase and a synergistic action of an α-amylase and a glucoamylase for efficient degradation of raw starch [20]. Apparently, the precursor enzyme was more active on raw starch compared to the 71-kDa glucoamylase since it possessed both these activities.

A. niger has been shown to secrete two glycosylated forms of glucoamylase G1 and G2 with molecular masses of 71 and

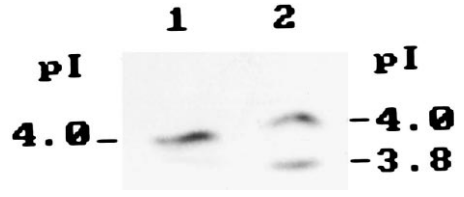

Fig. 6. Zymograms of raw starch activity in isoelectric focusing gels. Lane 1, purified 125-kDa precursor enzyme; lane 2, crude culture filtrate containing the precursor and 71-kDa glucoamylase proteins. The reducing sugars released from raw granular starch suspension (1 g in 75 ml 0.01 M sodium acetate buffer, pH 4.2) were visualised by staining with 2,3,5-triphenyltetrazolium chloride reagent [8].

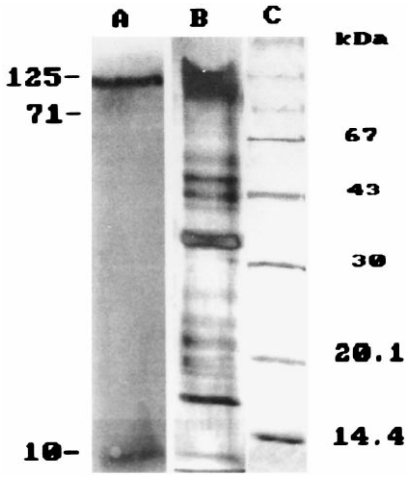

Fig. 7. Identification of 10-kDa non-enzymatic protein in the Western blots (A) of *A. niger* (CFTRI 1105). B: Silver-stained culture filtrate proteins. The occurrence of this protein in the culture filtrates was rare and could be identified in only a few experiments. On 12% SDS–PAGE gels, the resolution of other enzymatic proteins was reduced due to poor separation. C: Marker proteins.

61 kDa, respectively [21–23]. Both forms encoded by a single gene were immunologically related and possessed the same amino-terminal amino acid sequence [24]. The region of the G1 C-terminus containing about 100 amino acids absent in the G2 form has also been reported as the raw starch-binding domain [25]. Partial raw granular starch hydrolysing activity of the precursor identified in the 71-kDa glucoamylase, antibody cross-reactivity described above supported by the N-terminal sequence:

R
A-T-L-D-S-W-L-S-N-E-A-K-V-A-R-?-A-I-L-S-N-W
A

evidenced the glucoamylase as the N-terminal portion of the precursor since this precursor sequence was identical to that described from G1 (71 kDa) and G2 (61 kDa) forms of *A. niger*, *Aspergillus awamori* and *Aspergillus kawachi* glucoamylases. Cross-reactivity of the 53-kDa protein with the antibodies raised against the precursor only suggested that this enzyme is a proteolytically processed C-terminal fragment of the precursor amylase. The N-terminal A-T-P-A-D sequence obtained from the 53-kDa protein showed sequence similarity to the Taka amylase of *Aspergillus oryzae* [26]. The in vivo post-translational proteolytic processing of the 71-kDa form as reason for the occurrence of the 61-kDa glucoamylase in *A. niger* [3] could also be substantiated by the identification of a 10-kDa non-enzymic protein in some culture filtrates of *A. niger* using the antibody (Fig. 7). To ascertain the in vivo proteolytic processing causing the secretion of different amylases described above, experiments were carried out to identify amylase proteins in the fungal cell. Western blot analysis of protein extracts of protoplasts showed the presence of only the 125-kDa precursor enzyme (Fig. 8). This suggests proteolysis of the precursor in the periplasmic space. Most reports have shown the localisation of proteases in cell walls of *A. niger* [27–29] and the detection of the 10- and 53-kDa proteins in the culture filtrates explains their secretion without the need for a signal peptide. Since the cell walls of *A. niger* are less pervious to high molecular mass proteins [30,31], the large precursor protein is apparently proteolytically processed in the fungal periplasmic space for effective secretion to degrade the extracellular starch for growth. Hence, isolation of less sporulant and fast growing *A. niger* mutants that overproduced the 125-kDa precursor enzyme and the 71-kDa glucoamylase (Fig. 9) suggested deformities in cell wall structure and reduced or increased protease production.

Thus, it appears that the single chromosomal glucoamylase gene of *A. niger* described by Boel et al. [24] may be a larger gene encoding the precursor enzyme protein, the cellular regulation of which results in the secretion of glucoamylases and α-amylase by the fungus. This probably explains the absence

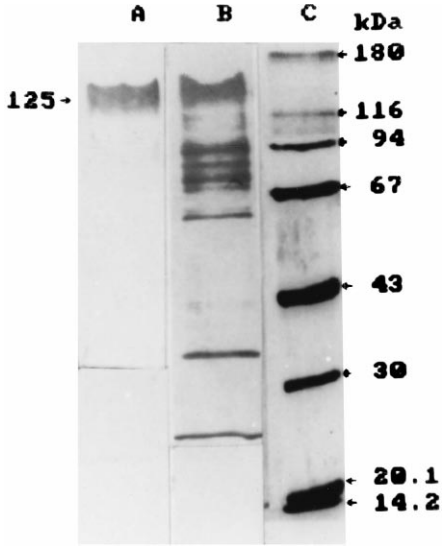

Fig. 8. Occurrence of only 125-kDa precursor enzyme in the protein extracts of *A. niger* protoplasts. The precursor protein was identified on Western blots (A) using antibodies after electrophoretic separation of (B) crude protein extracts of protoplasts in 10% SDS–PAGE gels. C: Marker proteins.

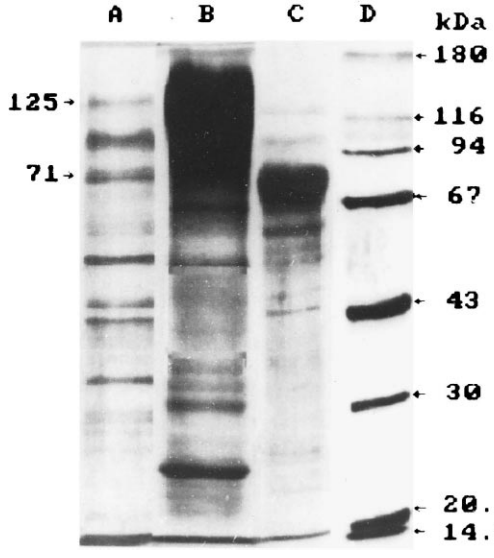

Fig. 9. SDS–PAGE of culture filtrate proteins of *A. niger* mutants (CFTRI 1105-41; CFTRI 1105-43) showing (B) hyperproduction of the 125-kDa precursor amylase and (C) 71-kDa glucoamylase. (A) and (D) correspond to culture filtrate proteins of the parent and marker proteins, respectively.

of initiation or termination of transcription or proper splicing of the intervening sequences of the *A. awamori* glucoamylase gene in *Saccharomyces cerevisiae* despite striking similarities of the consensus sequences within 5′- and 3′-flanking regions, as well as within the intervening sequences of the structural gene in yeast [32].

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