Evidence that cleavage of the precursor enzyme by autocatalysis caused secretion of multiple amylases by *Aspergillus niger*

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Abstract The observation that a mutant strain of *Aspergillus niger* isolated for protease overproduction accumulated Taka-amylase supported an earlier report that processing of the precursor amylase by protease resulted in the secretion of multiple amylases. Studies using a mutant strain revealed that such processing was not due to aspergillopepsin but to autocatalysis by an inherent protease activity of the precursor and glucoamylase. Alignment of protease sequences with glucoamylase showed regions of consensus with serine carboxypeptidase of *A. niger*. Thus point mutations in this region due to ultraviolet radiation apparently caused the mutant to evolve with enhanced protease activity that degraded the precursor and accumulated Taka-amylase.

Key words: Glucoamylase; Taka-amylase; Carboxypeptidase; Precursor; *Aspergillus niger*

1. Introduction

Amylases (EC 3.2.1.1) are a class of enzymes that hydrolyse starch to yield low molecular weight dextrins and sugars [1]. The importance of amylases in industry has been well documented over several years. Several amylases characterised from microbial sources have unique properties with specific applications for the production of various industrial products from starch [2].

Amylases reported from bacteria are usually single protein species with typical properties. However, multiple amylases have been described from the species of *Aspergillus* [1]. *Aspergillus niger* has been reported to produce two forms of glucoamylase [3], α-amylase [4], starch-hydrolysing enzyme [5], α-glucosidase [6] and Taka-amylase [7]. Despite the above reports, the literature is not clear on the mechanism of multiple amylase production by *A. niger*.

Cloning and sequence analysis of the glucoamylase gene of *A. niger* identified differential splicing of the glucoamylase mRNA for the secretion of G1 and G2 forms of the enzyme [8,9]. Studies using the peptide fragments obtained from G1 and G2 glucoamylases of *A. niger* suggested post-translational proteolysis in the C-terminal region of G1 [10]. Apparently, such a proteolysis was also responsible for the generation of multiple forms of amylases in *A. awamori*, *A. niger*, *A. saito* and *Rhizopus* species. Based on N-terminal amino acid sequence homology of the starch-hydrolysing enzyme protein of *A. niger* with the G1 and G2 forms of glucoamylase, cross-reactivity of the antibody raised to starch-hydrolysing enzyme with G1 and G2 glucoamylases and identification of raw starch-hydrolysing activities in the starch-hydrolysing enzyme and the G1 form of glucoamylase reasoned starch-hydrolysing enzyme as the amylase precursor from which the G1 and G2 forms of glucoamylase and Taka-amylase arose by proteolytic processing [7]. However, a protease involved in such a processing has not been characterised until now. In this study, a protease activity inherent to the starch-hydrolysing enzyme and the G1 form of glucoamylase of *A. niger* was identified. This activity evidenced the production of multiple amylases by the fungus due to autocatalytic cleavage. The details are described in this paper.

2. Materials and methods

2.1. Selection of mutant

A. niger used in this study is an industrial strain isolated for glucoamylase production [3] and maintained in this laboratory under accession number CFTRI 1105. A protease-overproducing mutant of this fungus was obtained after UV irradiation.

A young spore suspension of the parent culture was placed on a medium containing peptone (2%), dextrose (2%), yeast extract (1%) and agar-agar (2%). The plates were inverted on a transilluminator (302 nm) and mutation was carried out to obtain 60% population that survived the UV light treatment. After incubating the plates at 4°C in the dark, temperature selection was carried out by keeping the plates at 42°C for 24 h. The surviving colonies that emerged after incubating the plates at 30°C for 24 h were picked on to gelatin (gelatin 2%, dextrose 2%, yeast extract 1% and agar-agar 2%)-containing plates for screening. Presumptive colonies showing higher protease activity were identified by larger zones of clearance of gelatin. Further selection of mutants was done by assays for glucoamylase and Taka-amylase respectively. For this, the mutants were grown for 72 h at pH 5.5 in a broth containing corn starch (10%), peptone (1%) and yeast extract (0.5%).

2.2. Enzyme assays

Culture filtrates free of spores or hyphal fragments obtained after growth of the fungus by filtration through Whatman No. 1 filter paper were used for enzyme assay. Amylase assays were carried out using Lintner's soluble starch (BDH Laboratories, Poole, UK) or maltose prepared in 0.1 M sodium acetate buffer (pH 4.3) as substrates. Reducing sugars and glucose released were measured using 3,5-dinitrosalicylic acid [11] and the glucose oxidase–peroxidase reagent [12] respectively. The reaction mixture for the starch-hydrolysing enzyme and the glucoamylases contained 1 ml 2% maltose and 1 ml of appropriately diluted enzyme. The reaction was stopped after 30 min incubation with 3 ml 1.66 M Tris–HCl buffer (pH 7.6). The released glucose was estimated by the glucose oxidase–peroxidase method. Enzyme activity corresponded to μmol of maltose degraded

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per min. The assay for Taka-amylase was carried out at 40°C using 5 ml 2% soluble starch as substrate and 1 ml appropriately diluted enzyme. Preliminary experiments showed that acarbose (Bayer Pharmaceuticals, Mumbai, India) inhibited other interfering amylases but not the Taka-amylase activity. Hence for Taka-amylase assays, the culture filtrate was diluted 1:2 with acarbose (0.125%) containing 0.1 M sodium acetate buffer (pH 4.3) before use. The reaction was stopped after 30 min with 1 ml sodium hydroxide (4 M) and reducing sugars released were quantified as maltose equivalents using the 3,5-dinitrosalicylic acid reagent. The enzyme activity corresponded to μmol of maltose released per min. Protease activity of the culture filtrate was assayed at 30°C using 2% Hammarsten grade casein (pH 3.5) as a substrate. The reaction mixture contained 1 ml substrate and 200 μl of enzyme and after incubating it for 46 h, the amount of tyrosine released was measured using the Folin-Ciocalteau phenol reagent [13]. The unit activity corresponded to 1 μmol of tyrosine equivalents released per min. The protein content was measured using the Bradford reagent [14]. For some of the assays, the culture filtrate proteins were subjected to 60 and 90% ammonium sulphate fractionation. After separating the precipitated proteins by centrifugation ($5000\times g$ for 20 min) at 4°C, they were dissolved in sodium acetate buffer (0.02 M, pH 4.0) and dialysed against the same buffer at 4°C.

2.3. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 10% acrylamide gels. For some of the experiments, to improve protein separation and resolution, two-dimensional SDS–PAGE was carried out. After the first electrophoretic run in 10% polyacrylamide gels at 100 mA using Tris-glycine buffer (0.025 M, pH 8.8), the lane containing the separated proteins was cut and used for the second electrophoretic run. 10% SDS–polyacrylamide gel was cast after placing the lane horizontally and electrophoresis performed as above. The proteins in gels were visualised after silver staining [15].

Enzyme zymograms for protease and amylase activities were performed after separating the proteins by SDS–PAGE under non-reducing conditions in 10% acrylamide gels containing 0.1% gelatin or 0.1% Lintner's soluble starch respectively. After electrophoresis, for starch activity, the gels were washed in 0.1 M acetate buffer (pH 4.3). The gels were incubated at room temperature for 30 min and stained with iodide/iodine solution [7]. For protease activity zymograms [16], SDS was removed from the gels by incubation for 1 h in 0.02 M acetate buffer (pH 4.0) containing 2.5% Triton X-100. The protein was renatured by incubating the gels in the buffer for 5 h at 30°C. Gels were fixed and stained for 30 min with 0.1% amido black solution made in methanol:acetic acid:water (30:10:60). Protease activity was identified as zones of clearance after destaining the gels in the same solution without amido black [16].

2.4. Immunological methods

Antibodies were raised in rabbits by injecting 10 μg of protein [14] via the intramuscular route with Freund's complete adjuvant on days 0, 14, 28 and 42 [17]. Antiserum was collected by marginal ear vein bleeding. For Western blot analysis, proteins separated in 10% SDS–polyacrylamide gels and electroblotted on to nitrocellulose membranes [18] were used. The membranes were blocked with 15% skim milk solution and proteins were identified using antibody (1:500 dilution) and anti-rabbit goat IgG tagged with peroxidase (1:1000 dilution in 10m M Tris–HCl buffer saline, pH 8.0; Sigma, St. Louis, MO, USA). Tris–HCl buffer (50 mM, pH 7.6) containing hydrogen peroxide (0.025%) and 4-chloro-1-naphthol (0.04%) was used as substrate for the enzyme reaction. For some of the experiments, antibodies raised previously to the 125 kDa starch-hydrolysing enzyme [7] were also used.

3. Results and discussion

Of the five amylases secreted by *A. niger* in culture (Fig. 1), the 125 kDa starch-hydrolysing enzyme [5], 71 and 61 kDa glucoamylases [10] and 53 kDa Taka-amylase [7] hydrolysed starch at pH 4.3 to glucose and maltose (Taka-amylase). The fungus also produced a 53 kDa endo-active α-amylase that dextrinised starch at pH 6.0 [4]. The production of multiple

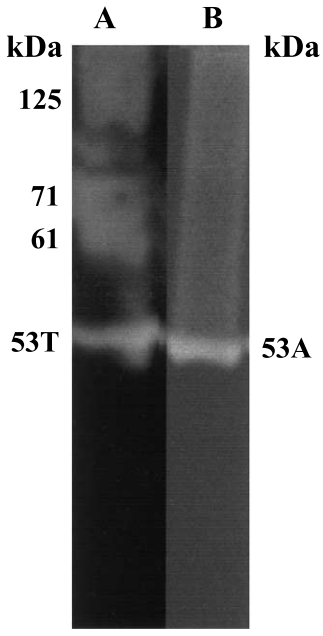

Fig. 1. Zymograms of amylases of *A. niger* (CFTRI 1105). The crude culture filtrate proteins (9 μg) were separated by SDS–PAGE in gels containing 0.1% Lintner's soluble starch under non-reducing conditions. After electrophoresis the gels were incubated in 0.1 M sodium acetate buffer of pH 4.3 (A) or pH 6.0 (B) for 30 min. Activity was visualised as zones of clearance after staining with iodide/iodine solution. 53T: Taka-amylase, 53A: α-amylase.

forms of the enzyme active at pH 4.3 apparently occurred by the proteolytic processing of precursor high molecular mass starch-hydrolysing enzyme [7] to overcome secretional difficulties for substrate utilisation due to reduced porosity of *A. niger* cell walls to high molecular mass proteins [19,20]. In this study, a mutant of *A. niger* affected in the general secretory pathway isolated by selection for temperature tolerance [21] also showed five-fold increased protease activity compared to the parent when grown in the medium containing starch (0.33 U/mg v. parent 0.066 U/mg). Growth of the mutant in the same medium showed accumulation of Taka-amylase (202 U/mg v. parent 130 U/mg) and a concomitant decrease of the other amylases active at pH 4.3 (12 U/mg v. parent 45 U/mg). This favoured the hypothesis that proteases have a role in amylase processing. Two-dimensional SDS–PAGE analysis for the amylases of the mutant and parent strains of the fungus also showed degradation of the 125 kDa starch-hydrolysing enzyme and accumulation of glucoamylases and the 53 kDa Taka-amylase (Fig. 2A,B). Thus it appeared that for efficient utilisation of starch substrate and since the cell walls of *A. niger* were less permeable to high molecular mass proteins, the precursor starch-hydrolysing enzyme was proteolytically processed to lower molecular mass 53 kDa Taka-amylase and 71 and 61 kDa glucoamylases (Fig. 2A,B).

Aspergillopepsins have been reported to be the major proteases produced by *A. niger* and the mutants affected in aspergillopepsin production were shown to overproduce glucoamylase in culture [22]. In the culture filtrates of the mutant strain of *A. niger*, zymogram analysis identified a clear 45 kDa protease (Fig. 3) which was apparently an aspergillopepsin due to similarity of the molecular mass reported of the enzyme. However, this protease activity was characteristically

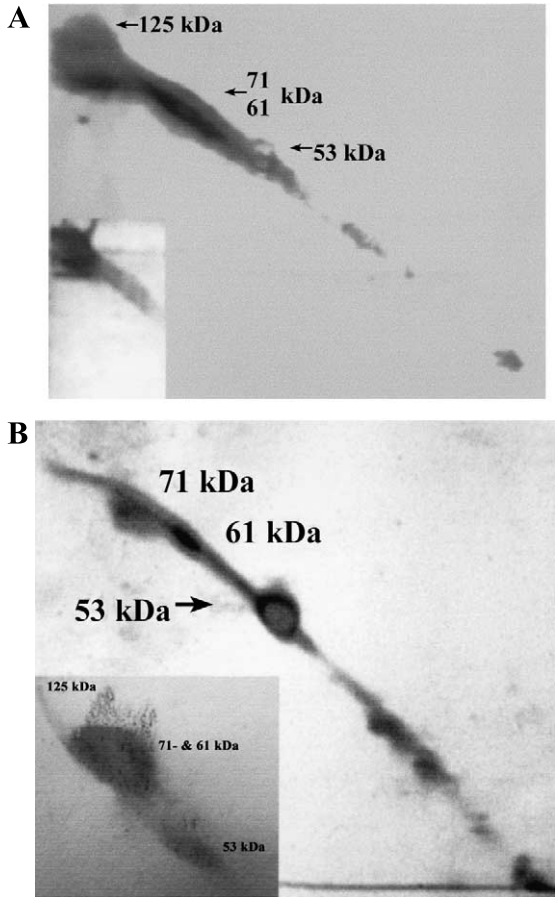

Fig. 2. Silver-stained two-dimensional SDS–PAGE of total culture filtrate proteins (15 μg) and their corresponding Western blot reactions (inset). The proteins from the culture filtrates of parent (A) and mutant (B) strains of *A. niger* grown in corn starch medium were separated by two-dimensional SDS–PAGE and probed with antibody raised against the precursor starch-hydrolysing enzyme.

absent in the culture filtrate of the parent (Fig. 3). Since the parent strain also produced multiple forms of amylase despite characteristic absence of aspergillopepsin in the culture filtrate, the results suggested that the higher protease activity

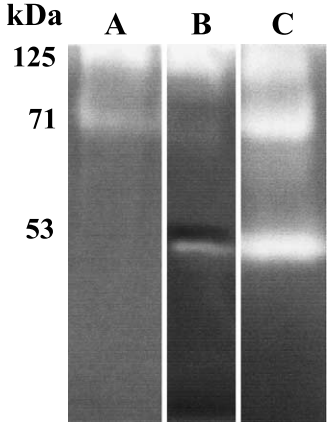

Fig. 3. Zymogram analysis. The culture filtrate proteins (5 μg) of the parent and mutant strains of *A. niger* were analysed for protease and amylase activities after SDS–PAGE in gels containing gelatin or starch. A: Protease activities in the parent. B: Protease activities in the mutant. C: Amylase activities in the mutant.

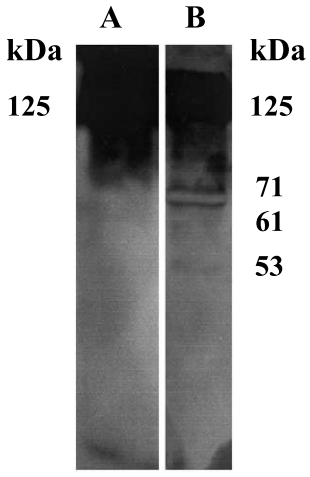

Fig. 4. Autocatalytic degradation of the precursor starch-hydrolysing enzyme (10 μg) during storage as identified by Western blot reactions. The purified precursor starch-hydrolysing enzyme (A) was stored at 4°C for 7 days in sodium acetate buffer (0.02 M) and analysed for degradation (B) using homologous antibody. Overloading of the protein in the wells was necessary to visualise the degraded products. Overloading affected protein transfer for Western blot reaction.

assayed in the culture filtrate of the mutant strain was due to overproduction of aspergillopepsin and that this protease had no role in the degradation of starch-hydrolysing enzyme (Fig. 2B). Zymogram analysis also showed the occurrence of protease activities in the regions corresponding to 125 kDa starch-hydrolysing enzyme and the 71 kDa G1 form of glucoamylase (Fig. 3). We could not reason this protease activity to co-migrate with the two amylolytic enzymes because, in the earlier study, a clear N-terminal amino acid sequence was obtained of the 125 kDa starch-hydrolysing enzyme by microsequencing [7]. To reason the inherent protease activity in starch-hydrolysing enzyme as a cause for its degradation, the purified enzyme of the parent strain was incubated at 4°C and analysed for the degraded products by performing Western blot reactions using homologous antibody. After 7 days incubation, degradation of protein and formation of the glucoamylase and Taka-amylase was observed (Fig. 4).

Ammonium sulphate precipitation of the culture filtrate

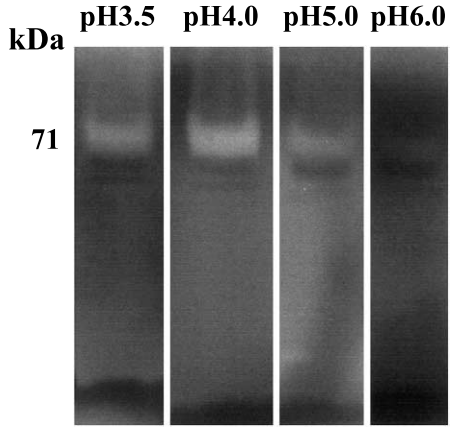

Fig. 5. Effect of pH on protease activity as determined by zymogram analysis. The gels were incubated at different pHs to visualise the activity. Identical protein concentrations (16.93 μg) were used in all lanes.

proteins of the mutant strain of *A. niger* separated aspergillopepsin from the amylases. While aspergillopepsin and Taka-amylase precipitated at 60% ammonium sulphate concentration, increasing the concentration to 90% precipitated other amylases in the supernatant. The precipitated amylases when assayed for protease by zymogram analysis showed a prominent activity at pH 4.0 in the 71 kDa glucoamylase protein (Fig. 5). Since this activity could explain the degradation of the 71 kDa glucoamylase to the 61 kDa enzyme, in vitro assay for protease was carried out by incubating the 60–90% ammonium sulphate fraction dissolved in sodium acetate buffer at room temperature. Despite 48 h incubation, no protease activity was detected.

Due to the absence of an easy assay for protease activity, the zymogram analysis described above was used as a means to purify the proteolytic enzyme by electroelution from the gels after preparatory SDS–PAGE. Antibodies raised to the purified enzyme reacted with 125, 71 and 61 kDa proteins in a Western blot analysis. The purified protein also showed both protease and amylase activities in zymogram reactions (Fig. 6). Antibody cross-reactivity and detection of protease activities in the starch-hydrolysing enzyme and the 71 kDa glucoamylase described above evidenced processing of the precursor amylase by the inherent protease to give rise to the 71 kDa G1 form of glucoamylase and the 53 kDa Taka-amylase. Further processing of the G1 form by the inherent protease apparently caused the formation of the 61 kDa G2 form of the enzyme. Absence of protease activity in the G2 form and Taka-amylase made these enzymes stable products of amylase protein processing in *A. niger*. The observation that the mutant strain of the fungus had a more pronounced protease activity in the G1 form compared to the parent strain reasoned more degradation of the starch-hydrolysing enzyme for the accumulation of Taka-amylase.

Though the identification of protease activities in the two amylolytic proteins explained autocatalysis as the cause for multiple amylase secretion by the fungus, it has also to reason the evolution of higher protease activity in the mutant strain that caused accumulation of Taka-amylase. Since the mutant strain of the fungus was isolated by treatment with UV radiation, the cause for enhanced protease activity in the amylases

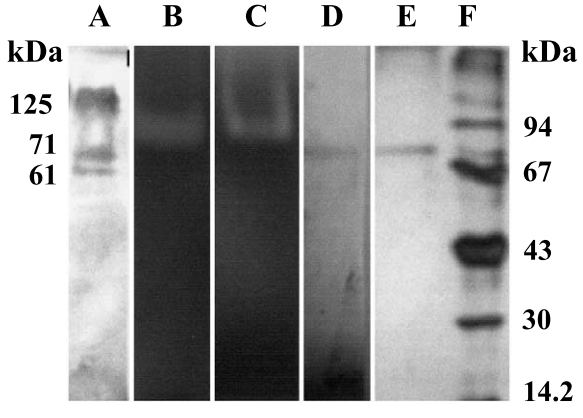

Fig. 6. Reactions of the electroeluted 71 kDa protein. A: Western reactions of the antibody raised to the purified enzyme (E: silver stained (3 μg); D: homologous reaction) with amylases in the crude culture filtrate of the parent (6 μg). B,C: Zymograms of the purified enzyme (3 μg) showing protease (B) and amylase (C) activities. Electrophoresis of the protein in substrate-containing gels caused migrational shifts. F: Molecular mass standards.

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Gl 188 SYVAQYWNQTG 198
Sp 156 SYAGMYVPYIA 166

Gl 389 SSSSTYSSIVDAVKTFADGFVSIVETHAASNGSMSEQYDK 428
Sp 337 SSWGPLPSVIERTNNTIIGH-GWLDYLLFLNGSLATIQNM 375
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Fig. 7. Multalin alignment of the serine type carboxypeptidase I sequence (Sp) of *A. niger* with the glucoamylase G1 protein sequence (Gl). Letters in bold indicate the catalytic amino acids of serine type carboxypeptidase I. Consensus regions are underlined.

can only be due to point mutations in the amylase gene. An alignment of the G1 glucoamylase with several protease sequences available at the NCBI using Multalin version 5.4.1 [23] has been made, and this showed that there were consensus regions (Fig. 7) within the G1 enzyme sequence with a serine type carboxypeptidase I (EC 3.4.16.-) of *A. niger* (NCBI accession number S78072 GI:7428206). This could indicate that the G1 enzyme has proteolytic activity.

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