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# Purification, Characterization, and Partial Primary Sequence of a Major-Maltotriose-producing α-Amylase, ScAmy43, from *Sclerotinia sclerotiorum*

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**A novel α-amylase (α-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1), ScAmy43, was found in the culture medium of the phytopathogenic fungus *Sclerotinia sclerotiorum* grown on oats flour. Purified to homogeneity, ScAmy43 appeared as a 43 kDa monomeric enzyme, as estimated by SDS-PAGE and Superdex 75 gel filtration. The MALDI peptide mass fingerprint of ScAmy43 tryptic digest as well as internal sequence analyses indicate that the enzyme has an original primary structure when compared with other fungal α-amylases. However, the sequence of the 12 N-terminal residues is homologous with those of *Aspergillus awamori* and *Aspergillus kawachii* amylases, suggesting that the new enzyme belongs to the same GH13 glycosyl hydrolase family. Assayed with soluble starch as substrate, this enzyme displayed optimal activity at pH 4 and 55°C with an apparent $K_m$ value of 1.66 mg/ml and $V_{max}$ of 0.1 μmol glucose·min$^{-1}$·ml$^{-1}$. ScAmy43 activity was strongly inhibited by $Cu^{2+}$, $Mn^{2+}$, and $Ba^{2+}$, moderately by $Fe^{2+}$, and was only weakly affected by $Ca^{2+}$ addition. However, since EDTA and EGTA did not inhibit ScAmy43 activity, this enzyme is probably not a metalloprotein. DTT and β-mercaptoethanol strongly increased the enzyme activity. Starting with soluble starch as substrate, the end products were mainly maltotriose, suggesting for this enzyme an endo action.**

**Keywords:** α-Amylase, biochemical characterization, maltotriose, *Sclerotinia sclerotiorum*

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α-Amylases (E.C. 3.2.1.1) are glycoside hydrolases that have been classified within family GH13 [12]. They play an important role in starch degradation and represent about 25 to 33% of the enzyme world market, in second place after proteases. They specifically catalyze the endohydrolysis of 1,4-α-D-glucosidic linkages of starch and related polysaccharides to produce oligosaccharides of different sizes.

α-Amylases constitute an important class of enzymes that find many biotechnological applications in processes requiring the degradation of starch [24], such as baking, brewing, deterging [13], and fabric desizing (in textile industries). Most α-amylases produce glucose or maltose as the major end products of starch hydrolysis. However, amylases that specifically produce maltooligosaccharides from starch have been reported [42]. These include maltohexaose-producing amylases from *Bacillus stearothermophilus* US100 [6] and *Bacillus amyloliquefaciens*; maltopentaose-producing amylase from *Bacillus cereus* NY-14 [43]; maltotetraose-producing amylases from *Pseudomonas stutzeri* [31] and *Bacillus circulans*, and maltotriose-producing amylases from *Streptomyces griseus* NA-468, *Bacillus subtilis* [40], and *Microbacterium imperiale* [39].

A number of amylases have been reported with different molecular weights, optimum pH, thermostability, and metal ion dependence [21, 23, 25, 27]. Amylases are universally produced by prokaryotes and eukaryotes, including plants and animals. However, only a few species of fungi and bacteria are used for the commercial production of α-amylases because of their ease of cultivation and the desirable physicochemical properties of the secreted enzymes [34, 36]. Filamentous fungi possess a number of features

that make them attractive for scientific as well as industrial purposes.

Among them, *S. sclerotiorum* displays a strong amylase activity when this microorganism is grown on oats flour. In fact, this fungus is a ubiquitous devastating plant pathogen, hugely destructive of many economically important crops including grain legumes and oilseed. *S. sclerotiorum* is well-known for the production of cell walls degrading enzymes [30] that have been purified and characterized, some of which having biotechnological uses, including endopolygalacturonases, β-xylosidases [3], β-glucosidases [37], xylanases [10], glucoamylases [21], and proteases. These enzymes facilitate the invasion and colonization of the infected host [29], particularly in diseases characterized by tissue maceration [5].

In the present study, we report the production, purification, and physicochemical and biochemical characterizations of a new extracellular α-amylolytic enzyme produced by the phytopathogenic fungus *S. sclerotiorum* when grown on oats flour as the carbon source. Moreover, we describe for the first time a new fungal enzyme giving mainly maltotriose as end products. ScAmy43 can be used for the production of this interesting substrate, which possesses wide applications in food processing.

## Materials and Methods

### Microorganism and Culture Conditions

*S. sclerotiorum* was obtained from the national fungi collection of the laboratory of cryptogamy INRAT-TUNISIE. The fungus was maintained on potato dextrose agar at 4°C. For enzyme production studies, *S. sclerotiorum* was grown in minimal liquid medium [20] containing 2% (w/v) glucose or different cereal flours as carbon sources: oats, wheat, or barley. The minimal medium also contained 1 g/l KCl, 0.5 g/l $MgSO_4$, 1 g/l $KH_2PO_4$, 1.4 g/l $NaNO_3$, 4 g/l $(NH_4)_2SO_4$, 2 g/l yeast extract, and 1 ml/l trace elements solution comprising 2 mg/l $CoCl_2$, 1.6 mg/l $MnSO_4 \cdot H_2O$, 1.4 mg/l $ZnSO_4 \cdot H_2O$, 5 mg/l $FeSO_4 \cdot 7 H_2O$. The pH was adjusted to 5.5 with HCl before autoclaving. Cultures were inoculated with mycelial disks cut of 3-day-old colonies and incubated at 25°C with orbital shaking at 150 rpm for 15 days. Mycelia were then removed by filtration and centrifugation at 8,000 ×*g* during 30 min at 4°C. The supernatants were used as crude enzyme preparation.

### Enzyme Assay

α-Amylase activity was determined using a short time standard assay at 55°C for 30 min by measuring the release of reducing sugar from 1% soluble starch (w/v) as substrate in 25 mM sodium acetate buffer, pH 4. The amount of released reducing sugars was determined by the dinitrosalicylic acid method [22]. The D-glucose was used as standard and one unit of α-amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars, expressed in glucose equivalent, per minute under the specified conditions.

### ScAmy43 Purification

Oats flour crude extract was brought to 70% ammonium sulfate saturation and stored overnight at 4°C. Then, the pellet was collected by centrifugation at 13,000 ×*g*, 4°C, for 30 min and dissolved in 25 mM sodium acetate buffer, pH 4. This protein extract was applied onto a Sephacryl S-200-HR glass column (2.5×100 cm) equilibrated with 25 mM sodium acetate buffer, pH 4, at 0.2 ml/min flow rate.

Fractions (3 ml) were collected and screened for amylase activity. Active fractions were pooled and concentrated by lyophilization. The dry residue, dissolved in 25 mM ammonium bicarbonate buffer+0.1 M NaCl, pH 8, was loaded on a Superdex 75 FPLC gel filtration column (1.6×60 cm, Pharmacia Biotech) equilibrated at 1 ml/min flow rate with the same buffer. Gel filtration calibration kit proteins, 200 kDa to 14 kDa (Sigma-Aldrich, France) were used for molecular mass estimation. Proteins were detected at 220 nm using an UV detector (model 332, Kontron Instruments, Switzerland). Fractions (1 ml) were collected and those showing amylase activities were pooled, dialyzed, and concentrated by freeze-drying. The dry residue dissolved in 25 mM $NH_4HCO_3$ buffer, pH 8, was loaded on a quaternary methyl ammonium (QMA) Accel Plus AP1 column (1×20 cm; Waters, France). The column was eluted at a flow rate of 2 ml/min, with a linear NaCl gradient from 0 to 1 M in 25 mM $NH_4HCO_3$ buffer, pH 8. Active fractions were pooled and lyophilized before electrophoresis analysis.

### Protein Determination

The protein content was determined by the Bradford method [8], using a Bio-Rad assay (Bio-Rad), with bovine serum albumin (Sigma-Aldrich, France) as protein standard.

### Examination of Purity and Estimation of the α-Amylase Molecular Mass

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10% polyacrylamide gel as described by Laemmli [17]. Molecular mass markers (29 to 205 kDa; Sigma-Aldrich, France) were included and the gel was silver stained [7].

### ScAmy43 Biochemical Characterization

**Influence of Temperature and pH on Enzyme Activity and Stability.** Amylase activity was determined with the short time standard assay at various temperatures between 30 and 80°C in 25 mM sodium acetate buffer, for 30 min at pH 4, using soluble starch as substrate and the dinitrosalicylic acid method as described previously.

Thermostability was investigated by measuring the residual activity at 55°C, using the short time standard assay, after amylase incubation for 30 min at various temperatures between 30 and 80°C in the absence or in the presence of substrate. Optimum pH of the amylase was determined at 55°C with the standard assay at different pH values using the following buffers: 25 mM citrate-phosphate buffers (pH 2.5–7), 25 mM Tris-HCl buffers (pH 7.5–8.5), and 25 mM glycine-NaOH buffers (pH 9–11). pH stability was studied by measuring residual activity at pH 4, 55°C, after enzyme incubation at various pH values between 2.5 and 11 at 4°C for 24 h.

**Effects of Metal Ions, Detergents, and Reducing Agents on Amylase Activity.** Enzyme samples (0.5 ml) containing purified amylase, in 25 mM sodium acetate buffer, pH 4, were incubated at 4°C for 30 min with one of the following reagents: 5 mM of either $ZnCl_2$, $MgCl_2$, $CaCl_2$, $FeCl_2$, $MnCl_2$, $CuCl_2$, or $BaCl_2$; 6 M urea; 2% (w/v) SDS; 5, 10, or 20 mM of ethylenediaminetetraacetic acid (EDTA) or ethylene glycol-bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic

acid (EGTA), 5 mM dithiothreitol (DTT); or 5 mM β-mercaptoethanol (β-ME). Then, 0.5 ml of 1% (w/v) soluble starch in 25 mM sodium acetate buffer, pH 4, was added and the enzyme residual activity was measured as described above, using the standard assay without added reagent as 100%.

### ScAmy43 Catalytic Properties

**Enzyme Kinetic Measurements with Soluble Starch.** Using soluble starch as substrate, kinetic parameters of the purified α-amylase were investigated in 25 mM sodium acetate buffer, pH 4, at 55°C. The purified enzyme (7.5 U) was added to 1 ml of reaction mixture containing 1% soluble starch. The initial velocity was then determined and the steady state kinetic parameter, Michaelis constant ($K_m$), was calculated using a Lineweaver-Burk plot.

**Analysis of End Products by Normal Phase Chromatography.** Purified amylase (15 U) was added to 1 ml of 1% soluble starch in 25 mM sodium acetate buffer, pH 4, and then incubated at 40°C. After 2 h, 6 h, 24 h, 6 days and 12 days incubation times, enzymatic reaction was stopped by addition of 2 ml of absolute ethanol. The reaction mixture was centrifuged at 10,000 ×g for 10 min to remove undigested residual starch and the supernatant was concentrated under vacuum. The dried residue was dissolved in 0.15 ml of $H_2O$/Acetonitrile, 25/75 (v/v), and applied onto an aminopropyl Ultrasep-$NH_2$ column (250×4 mm; Bischoff, Germany). The column was eluted at 1 ml/min flow rate, 35°C, with $H_2O$/Acetonitrile, 25/75 (v/v). End products, mono- and oligosaccharides, were detected using a refractive index monitor (model 475; Kontron Instruments, Switzerland) connected to an acquisition data system (Millenium[32]; Waters) and their retention times were compared with those of standard saccharides (glucose, maltose, and maltotriose) determined under the same conditions. End products analysis was achieved using the external standard calibration method.

**Analysis of End Products by Thin Layer Chromatography.** Qualitative analysis of end products molecular size distribution, after 2 h to 12 days of ScAmy43 incubation times with soluble starch, was examined by thin layer chromatography (TLC) on silica gel plate, SilG-25 (20×20 cm; Macherey-Nagel, Germany). TLC was developed by the ascending chromatography technique in a solvent system of n-butanol/ethanol/water (2:1:1) at room temperature. Sugars were detected by spraying with 10% sulfuric acid in methanol, and then heating at 110°C for 10 min.

**Substrate Specificity.** To determine the substrate specificity of the enzyme, several gelatinized starches such as potato, maize, rice, potato soluble starch, and amylose (Sigma-Aldrich, France) at 1% (w/v) in 25 mM sodium acetate buffer, pH 4, were used as substrates for the purified enzyme during the assay. Released reducing sugars were assayed by the standard dinitrosalicylate procedure as described above with glucose as a standard.

### ScAmy43 Physicochemical Properties

**$NH_2$-Terminal Amino Acid Sequence.** After SDS-PAGE of the purified amylase on 12% polyacrylamide gel, electroblotting was performed on PVDF membrane (Millipore, France). The membrane was stained with Coomassie brilliant blue (0.1% in 50% methanol). The band corresponding to the purified protein was cut from the membrane and subjected to $NH_2$-terminal sequence analysis (Institut de Biologie et Chimie des Protéines, Lyon, France) by automated Edman degradation (model Procise 492A; Applied Biosystems).

### Internal Sequence Analysis by Mass Spectrometry

**In-Gel Proteolysis.** The stained purified ScAmy43 band was excised and washed with 50% acetonitrile in 50 mM ammonium bicarbonate, pH 8, and then sliced into small pieces and partially dried under vacuum. The gel pieces were rehydrated in digestion buffer, 100 mM ammonium bicarbonate, pH 8, containing 5 μg/ml trypsin [35]. Digestion was carried out for 4 h at 37°C and was stopped by addition of 60% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water. Peptide fractions were concentrated to a final volume of 10–20 μl for mass spectrometric analysis.

### Mass Spectrometry

**MALDI-TOF MS.** The peptide mixture (1 μl) was mixed with an equal volume of matrix solution (4 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% aqueous TFA) and deposited onto the MALDI target plate using a MALDI-Q-TOF Premier (Waters, Manchester, U.K.) instrument. External calibration covering the *m/z* 729–3,959 mass range [14] was achieved with a mixture of polyethylene glycols. A single point lock-mass (Waters) correction was used as reference (Glu-fibrinopeptide, *m/z* 1570.6774; Sigma-Aldrich) and was applied to all spectra. Calibrated spectra were submitted to database searches (Swissprot, NCBI) using the MASCOT mass mapping software.

**ESI-LC-MS/MS.** The tryptic peptide mixture was separated by reverse-phase capillary liquid chromatography on a C18 column (75 μm inner diameter; Waters, Manchester, U.K.) [9]. The column effluent (200 μl/min) was connected to the nanospray source of an ESI-Q-TOF Micro mass spectrometer equipped with a NanoLockspray system (Waters), using Glu-fibrinopeptide as reference. Data mining (Swissprot and NCBI databases) using tandem mass spectra produced by collision-induced dissociation (CID) of peptides was performed by means of the MASCOT MS-MS search algorithm.

## RESULTS AND DISCUSSION

### ScAmy43 Purification

In a preliminary study, we demonstrated a maximal amylase production using oats flour as substrate in the Mandel's medium. Consequently, amylase was purified from supernatants of *S. sclerotiorum* grown on oats flour.

Supernatant proteins were precipitated using 70% ammonium sulfate and the pellet obtained was dissolved in 25 mM sodium acetate buffer, pH 4.

After exclusion chromatography on Sephacryl S-200-HR, a single peak of α-amylase activity was obtained. Active fractions were applied onto a FPLC gel filtration Superdex 75 column. This step efficiently removed large amounts of high molecular mass contaminant proteins, as shown by SDS-PAGE analysis (Fig. 1, lane 3). The Superdex 75 active fractions were pooled and loaded onto an Accel Plus QMA anion-exchange column. Amylase activity was recovered in the unbound material, and SDS-PAGE analysis of the purified fraction revealed a single band (Fig. 1, lane 4), indicating that the amylase was purified

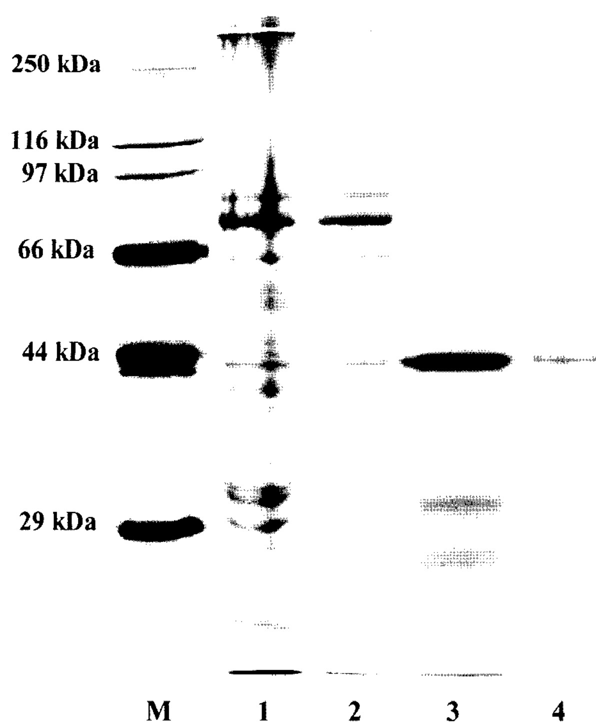


**Fig. 1.** Silver staining SDS-polyacrylamide gel electrophoresis of the purified ScAmy43 from *S. sclerotiorum*.

M: High molecular mass standard protein markers; lane 1: crude culture filtrate; lane 2: active fraction after Sephacryl S-200-HR; lane 3: active fraction after size exclusion chromatography on Superdex 75; lane 4: purified amylase after anion-exchange chromatography on QMA column.

to homogeneity and had a 43 kDa apparent molecular mass. Thus, the purified enzyme was designated ScAmy43.

Detailed studies on fungal α-amylase purification have been virtually limited to a few species of mesophilic fungi [16]. Our purification process was different and simpler than that employed by Abe *et al.* [1] for the purification of *Aspergillus* sp. α-amylase, which includes six steps.

## ScAmy43 Physicochemical and Biochemical Characterization

**Molecular Mass.** The 43 kDa molecular mass of ScAmy43, determined by SDS PAGE, is in good agreement with the 40 kDa molecular mass estimated by size exclusion chromatography, indicating that the purified enzyme is monomeric. Thus, the molecular mass of ScAmy43 is lower than those previously reported for amylases from other fungal sources: *S. sclerotiorum* 72 kDa [21], *Aspergillus* sp. 56 kDa, *Aspergillus oryzae* 50 kDa, *Aspergillus fumigatus* 65 kDa, and *Aspergillus flavus* 75 kDa [16].

**$NH_2$-Terminal Amino Acid Sequence.** Twelve amino acids were sequenced from the N-terminus of ScAmy43 and compared with those of other fungal amylases. The partial N-terminal sequence of ScAmy43 showed a high level of homology with those of α-amylase isolated from *Aspergillus kawachii*, *Aspergillus awamori* 3, and *Aspergillus niger* (86%), *Emericella nidulans* (74%), and

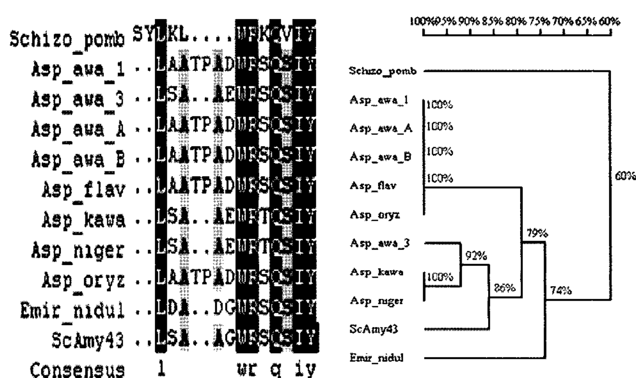


**Fig. 2.** N-terminal sequence alignment of ScAmy43 with other α-amylase sequences.

Amino acid residues conserved in all sequences are written in bold type. Sequence alignment was performed using the DNAMAN program (Version 5. 2. 2; Lynnon Biosoft, Que., Canada).

*Schizosaccharomyces pombe* (60%) (Fig. 2). All α-amylases listed in Fig. 2 belong to the GH13 glycosyl hydrolase family, suggesting that our enzyme also belongs to the same GH13 family [12].

**Internal Sequence Analysis.** In spite of an expected mass accuracy below 5 ppm currently obtained with lock-mass correction, direct protein identification could not be achieved from the MALDI mass fingerprint of the tryptic peptide mixture obtained from the ScAmy43 gel band (MASCOT, Matrix Science software; Uniprot and NCBI databases). None of the observed ScAmy43's tryptic peptide masses were identical to those obtained from other fungal α-amylases like *Aspergilus awamori*, *Asp. kawachii*, and *Asp. niger*. This observation suggests that the ScAmy43 primary structure is different from those of other fungal amylases belonging to the GH13 family.

The tryptic peptide mixture was then separated by reversed-phase nanochromatography and analyzed by means of ESI-MS/MS. The resulting set of collision induced dissociation (CID) mass data did not lead to protein identification either. Then, we undertook *de novo* sequencing on the best CID spectra, and used the resulting sequence tags (6 to 12 contiguous amino acids) for homology searches (BLAST). Only one peptide, identified as SXYQVX (where X stands for either leucine or isoleucine that cannot be differentiated by low-energy CID), revealed a high level of homology with peptide stretches belonging to fungal α-amylases. The two other internal sequences, respectively identified as XYVDPT and SNSNSYS[Q/K]SATY, did not present any homology with those of other fungal amylases. This observation suggests the primary structure originality of ScAmy43 α-amylase.

**Effects of pH and Temperature on ScAmy43 Activity and Stability.** The effect of pH on ScAmy43 activity and stability is shown in Fig. 3. The result was a bell-shaped curve showing an optimal activity at pH 4 (Fig. 3A). The activity was found to decrease gradually at higher and

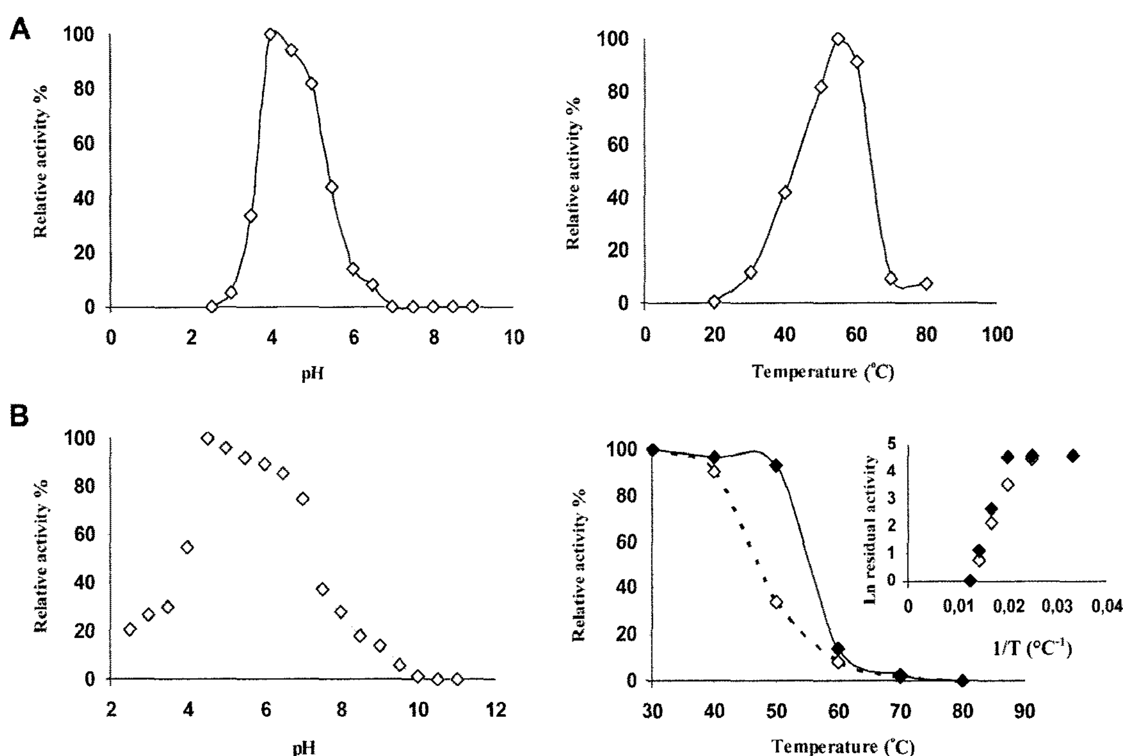


**Fig. 3.** Effects of pH and temperature on activity (**A**) and stability (**B**) of the purified ScAmy43.
Thermostability of ScAmy43 was determined by pre-incubation during 30 min at different temperatures in the presence (solid lines) or absence (dotted lines) of 1% soluble starch; after that, the remaining activity was measured using the standard short time assay. For pH stability testing, the enzyme was mixed with different buffers, and the mixture incubated for 24 h at 4°C. Thereafter, 0.5 ml of 1% (w/v) soluble potato starch in 25 mM sodium acetate buffer, pH 4, was added to the enzyme, which was then incubated at 55°C for 30 min. Residual enzyme activity was determined by the standard short time assay.

lower pH ranges, and ScAmy43 showed 50% of maximal activity at pH 3.5 and 5.5. The optimum pH of ScAmy43 was not different from those of other bacterial and fungal amylases [26]. ScAmy43 was stable over a narrow range of pH, between 4 and 7 (Fig. 3B).

The influence of temperature on the amylase activity and stability was also performed. Using the standard short time assay (30 min), the maximum activity of ScAmy43 was observed at 55°C (Fig. 3A). The activity increased sharply with gradual increase in temperature up to 55°C, and it gradually declined with further rise in temperature, indicating loss in the active conformation of the enzyme. The enzyme was only 15% active at 70°C. These results are in accordance with those previously reported for amy III, one of two allozymes of α-amylase from *S. oryzae* [2].

As shown in Fig. 3B, ScAmy43 was stable in the absence of substrate at temperatures up to 40°C for 30 min pre-incubation. Rapid inactivation occurred above 40°C and only 20% of maximal activity was detected at 55°C in the same conditions. When ScAmy43 thermostability was tested at 55°C, the enzyme was incubated at such a temperature during 60 min, including 30 min pre-incubation time, prior to the 30 min short time standard assay.

Thermodenaturation of ScAmy43 at a given temperature is time dependent, so enzyme inactivation at 55°C was higher after 60 min incubation time than that observed after only 30 min at the same temperature during the standard short time assay. That is why the α-amylase produced by *S. sclerotiorum* has optimal activity at 55°C when tested during a short time period but is not stable at the same temperature during a longer period. Such a phenomenon is reported for amylase produced by *Lactobacillus manihotivorans* LMG 18010 [4].

These enzymatic parameters are typical of the starch-hydrolyzing enzymes and especially the fungal amylases [11].

The presence of 1% (w/v) potato starch in the reaction mixture also enhanced amylase thermostability at temperature above 50°C, suggesting that the high starch concentration of some processes could have improved the performance of ScAmy43.

**Effects of Metal Ions and Chemical Reagents on ScAmy43 Activity.** Effects of divalent metal ions on the purified ScAmy43 activity was determined at pH 4 and 55°C. Results are given in Table 1. Metal ions such as $Cu^{2+}$, $Mn^{2+}$, and $Ba^{2+}$ at 5 mM dramatically decreased the amylase activity; however, the inactivation effect of $Fe^{2+}$ was found to be partial. We only observed a slightly positive effect of $Ca^{2+}$, $Zn^{2+}$, and $Mg^{2+}$ on ScAmy43 activity. Such divalent cations increased amylase activity from 3 to 11%. At the same time, α-amylase produced by *S.*

**Table 1.** Effects of metal ions and chemical reagents on ScAmy43 activity from *S. sclerotiorum*. All metal ions or chemical reagent were tested at the concentration of 5 mM except for Urea (6 M), SDS (2%), and EDTA and EGTA (5, 10, and 20 mM). The relative activity was determined by measuring the ScAmy43 activity at 55°C and pH 4 after pre-incubation at 4°C for 30 min in the presence of an individual metal ion or reagent. The activity assayed in the absence of metal ions or reagents was taken as 100%.

| Metal ions or reagents | Relative activity % |
|---|---|
| Control | 100 |
| $ZnCl_2$ | 104 |
| $MgCl_2$ | 103 |
| $CaCl_2$ | 111 |
| $FeCl_2$ | 85 |
| $MnCl_2$ | 44 |
| $CuCl_2$ | 26 |
| $BaCl_2$ | 25 |
| β-ME | 172 |
| DTT | 166 |
| EGTA | 100 |
| EDTA | 95 |
| Urea | 63 |
| SDS | 11 |

*sclerotiorum* was not sensitive to metal chelating agent addition, such as EGTA, or very slightly to EDTA (Table 1). Therefore, we can postulate that ScAmy 43 is probably not a metalloenzyme. These results are in accordance with those reported by Steyn and Pretorius [38], who characterized a novel α-amylase from *Lipomyces kononenkoae*. The new enzyme produced by the yeast was slightly stimulated by $Ca^{2+}$ (6%) but was not affected by 1 to 5 mM EDTA or EGTA additions.

Generally, α-amylases are known to be calcium metalloenzymes [28] and the role of $Ca^{2+}$ in maintaining the stability and structure of α-amylases has been demonstrated by Mishra and Maheshwari [23] and Ronaszéki *et al.* [32]. However, despite that most of the amylases are known to be metal ion dependent enzymes, the occurrence of $Ca^{2+}$-independent amylases have been reported in *Bacillus thermooleovorans* NP54 [19], *Bacillus acidocaldarius* [15], and *Bacillus brevis* [41].

The effects of other chemical reagents on the α-amylase activity were examined (Table 1). The enzyme was easily denatured by SDS and only partially by urea.

Reagents that cause reduction of disulfide bond, such as β-ME and DTT, strongly increased the enzyme activity. This result suggests that the DTT and β-ME effects could be the cause of the better accessibility of the substrate to the catalytic site after disruption of the interdisulfide bridge [18]. The presence of cysteine residues at the surface of *Bacillus subtilis* α-amylase leads to the formation of intermolecular disulfide linkages, leading to the aggregation of amylase monomer. This result has been described by Liu *et al.* [18] and explains why the addition of β-ME dramatically reduces the enzyme aggregate size by destroying the intermolecular disulfide bond, and hence triggering and enhancing of the α-amylase activity.

**Table 2.** ScAmy43 starch specificity. The reaction mixture consisted of 1% of various starches and 7.5 U/ml of purified ScAmy43 in 25 mM sodium acetate buffer, pH 4, at 55°C and for 30 min. Reducing sugars released were assayed by the standard dinitrosalicylate procedure. Soluble potato starch was taken as 100%.

| Substrate | Relative activity (%) |
|---|---|
| Soluble potato starch | 100 |
| Gelatinized amylose | 74 |
| Potato native starch | 0.2 |
| Potato gelatinized starch | 106 |
| Maize native starch | 0.8 |
| Maize gelatinized starch | 53 |
| Rice native starch | 0.5 |
| Rice gelatinized starch | 52 |

### ScAmy43 Catalytic Properties

**Kinetic Constants.** Apparent $K_m$ and $V_{max}$ values, determined from Lineweaver-Burk plots, were 1.66 mg/ml and 0.1 μmol glucose·min$^{-1}$·ml$^{-1}$, respectively, with soluble starch as substrate. It is difficult to compare these kinetic values with those of amylases obtained by other workers, because of the various origins of starch substrates and differences in assay conditions; however, the $K_m$ value, for soluble starch, of ScAmy43 falls within the same range as those of many fungal amylases [23, 25].

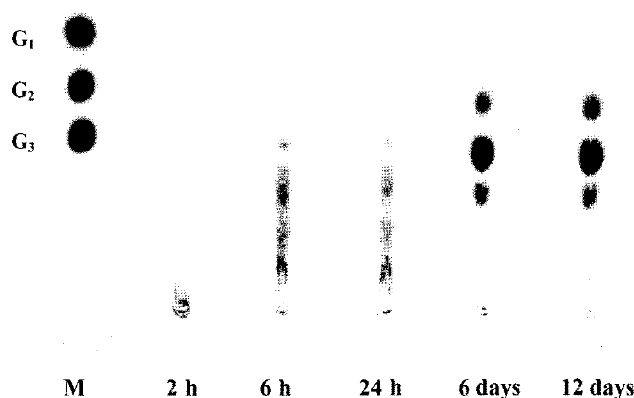


**Fig. 4.** Thin layer chromatography of the products of hydrolysis obtained from soluble starch, 1% (w/v), by purified ScAmy43.
The reaction was done at 40°C, pH 4.0, in 25 mM sodium acetate buffer. Samples were taken at different incubation times, the enzymatic reaction was stopped by addition of 2 vol of absolute ethanol, and then the reaction mixture was centrifuged to remove undigested residual starch. Supernatant was concentrated under vacuum before analysis. (M) standard mixture; glucose, $G_1$; maltose, $G_2$; maltotriose, $G_3$; hydrolytic products at 2 h, 6 h, 24 h, 6 days, and 12 days incubation time, respectively.

**Starch Specificity.** Purified ScAmy43 was examined for its ability to hydrolyze various native and gelatinized starches under standard assay condition and soluble potato starch hydrolysis was taken as the reference. *Sclerotinia sclerotiorum* α-amylase hydrolyzed efficiently gelatinized starch from potato, maize, rice, and amylose with a 106, 53, 52%, and 74% hydrolysis rate, respectively. ScAmy43 does not hydrolyze native starches (Table 2).

α-Amylase starch hydrolysis is greatly enhanced by gelatinization for 15 min at 100°C. In fact, upon further heating (pasting or cooking), amylose and portions of the amylopectin each from the granule produce a viscous suspension and the α-1,4 linkages will be more easily accessible to the enzyme. This fact explains why we obtained the same amount of hydrolysis percentage from both gelatinized potato starch and soluble potato starch.

## ScAmy43, a Major Maltotriose Producer

The action of purified ScAmy43 on soluble starch was examined. Hydrolytic products extracted with 66% (v/v) ethanol were examined qualitatively by thin layer chromatography (Fig. 4) and quantitatively by normal phase chromatography (Fig. 5). When soluble starch hydrolysis was complete, after 6 days incubation time, the ScAmy43 hydrolytic pattern consisted of a mixture containing maltotriose as the major end product, a trace of glucose, and small amounts of maltose and maltotetraose. Maltotriose

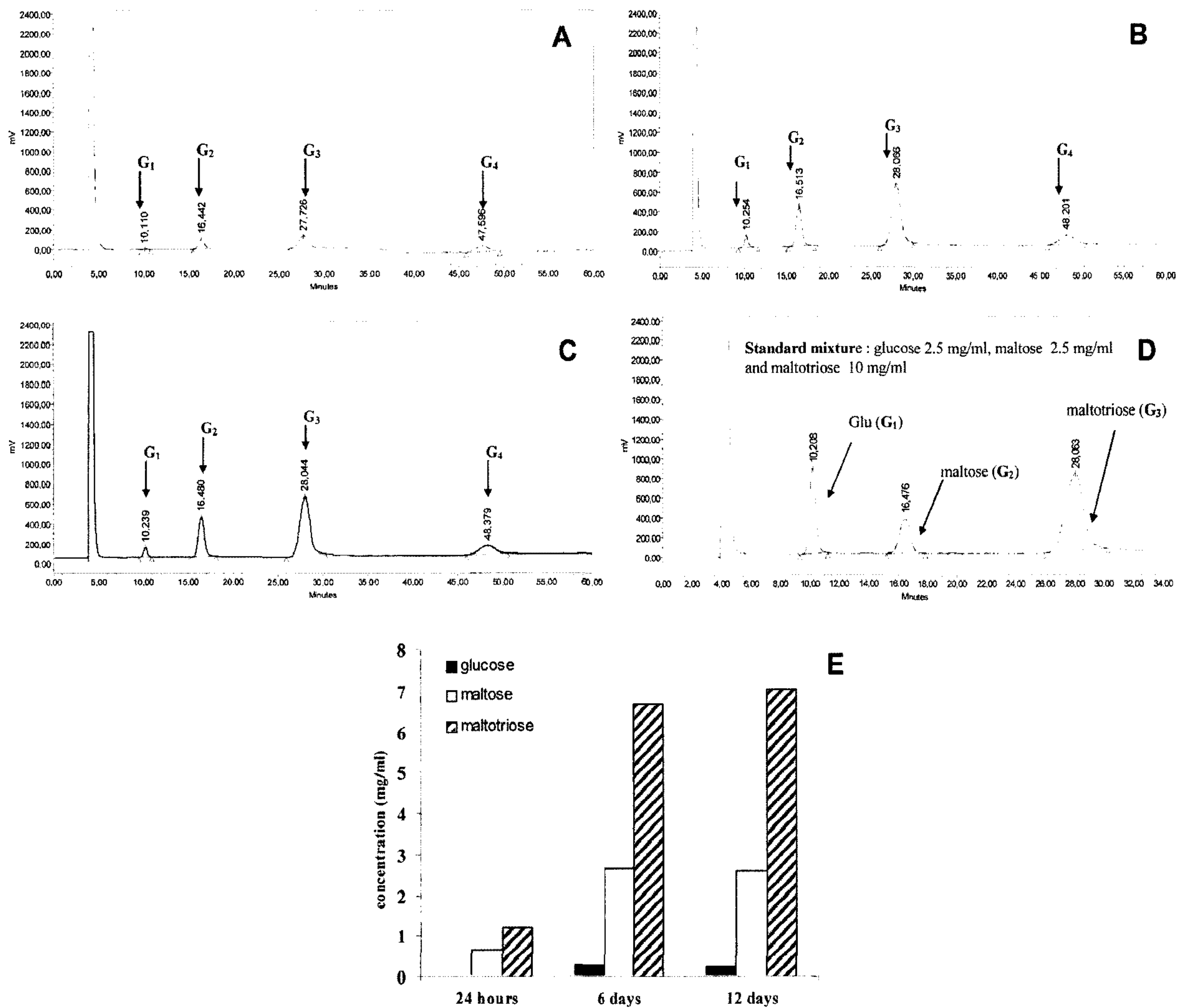


**Fig. 5.** Analysis of end products after soluble starch hydrolysis by the purified ScAmy43.

Hydrolysis of 1% soluble starch was done at 40°C, pH 4.0. Samples were then taken at different incubation times, enzymatic reaction was stopped by addition of 2 vol of absolute ethanol, and the reaction mixture was centrifuged to remove undigested residual starch. Supernatant was concentrated under vacuum before analysis of end products by normal phase high performance liquid chromatography on an amino-propyl Ultrasep $NH_2$ column. Monosaccharide and oligosaccharides were detected using refractive index monitoring and their retention times were compared with those of standard saccharides (glucose, maltose, and maltotriose) analyzed in the same conditions (**D**). **A**, **B**, and **C** correspond to end products distribution after 24 h, 6 days, and 12 days incubation times, respectively. **E.** Glucose, $G_1$; maltose, $G_2$; and maltotriose, $G_3$ were quantified in reaction products at 24 h, 6 days, and 12 days incubation times, with the external standard method, using the standard mixture chromatogram, (**D**) as reference.

represented 70% (w/w) of the end products including glucose + maltose + maltotriose (maltotetraose was not quantified) (Fig. 5E). The new fungal enzyme is an endo amylase, which cleaves the α-1,4 bonds in starch without releasing monomer, as was observed for other amylases secreted by *S. sclerotiorum* [21]. Such a result confirms that ScAmy43 can be considered as a major maltotriose-producing amylase. The amount of maltotriose produced by our fungal amylase during soluble starch hydrolysis was quite similar to those described for other maltotriose-producing amylases such as *Thermobifida fusca* amylase, 63% $G_3$ [42]; *Streptococcus bovis* amylase, 78% $G_3$ [33]; and *Bacillus subtilis,* 56% $G_3$ [40].

Maltotriose-producing amylases are essentially produced by bacteria, and to our knowledge, no fungal amylase producing maltotriose as the major end product had been described.

Maltotriose possesses many excellent properties for the food industry [42], including mild sweetness and the prevention of retrogradation of starch in foodstuffs.

An α-Amylase secreted by *S. sclerotiorum*, ScAmy43, purified by a three-step purification procedure, was found to consist of a 43 kDa monomeric protein. Molecular mass and ion requirement of the purified enzyme showed differences with other microbial α-amylases. Compared with other fungal α-amylases, ScAmy43 displays some interesting characteristics such as a low molecular mass and a starch hydrolytic pattern. The partial N-terminal sequence of the purified enzyme showed high similarity with fungal amylases, and suggests that this α-amylase enzyme also belongs to glycosyl hydrolase family GH13. However, the MALDI mass fingerprint of ScAmy43 tryptic digest, as well as internal sequences analyses, indicate that the structure of the α-amylase produced by *S. sclerotiorum* is unique when compared with other fungal amylases. The ability of the enzyme to produce maltotriose as the major end product makes ScAmy43 potentially useful for various biotechnological processes. We are currently working on the isolation of the *S. sclerotiorum* amylase gene, by using oligonucleotides as probe that correspond to the $NH_2$-terminal acid sequence and internal sequences identified for the new amylase. The cloning and characterization of the ScAmy43 gene from *S. sclerotiorum* will help us to elucidate the structure of this enzyme and the regulation of its production by this fungus.

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