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Structural Investigation and Homology Modeling Studies of Native and Truncated Forms of α-Amylases from *Sclerotinia sclerotiorum*

Ben Abdelmalek, Imen[1], Maria Camino Urdaci[2], Mamdouh Ben Ali[3], Muriel Denayrolles[2], Stephane Chaignepain[4], Ferid Limam[1,5], Samir Bejar[3], and Mohamed Nejib Marzouki[1]*

[1]*Institut National des Sciences Appliquées et de Technologie (INSAT), Centre Urbain Nord 676-1080 Tunis Cedex, Tunisia*
[2]*LMBA, UMR 5248 CNRS -U. Bordeaux 1- ENITAB, 2 rue Robert Escarpit, 33607 Pessac Cedex, France*
[3]*Centre de Biotechnologie de Sfax, BP «K» 3038, Sfax, Tunisia*
[4]*Institut Européen de Chimie et Biologie (IECB), UMR 5248 CNRS -U. Bordeaux 1ENITAB, 2 rue Robert Escarpit, 33607 Pessac Cedex, France*
[5]*Centre de Biotechnologie, Technopole Borj Cédria (CBBC), BP 109-2050 Hammam-Lif Cedex, Tunisia*

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The filamentous ascomycete *Sclerotinia sclerotiorum* is well known for its ability to produce a large variety of hydrolytic enzymes. Two α-amylases ScAmy54 and ScAmy43 predicted to play an important role in starch degradation were showed to produce specific oligosaccharides essentially maltotriose that have a considerable commercial interest. Primary structure of the two enzymes was established by N-terminal sequencing, MALDI-TOF masse spectrometry and cDNA cloning. The two proteins have the same N-terminal catalytic domain and ScAmy43 derived from ScAmy54 by truncation of 96 amino acids at the carboxyl-terminal region. Data of genomic analysis suggested that the two enzymes originated from the same α-amylase gene and that truncation of ScAmy54 to ScAmy43 occurred probably during *S. sclerotiorum* cultivation. The structural gene of Scamy54 consisted of 9 exons and 8 introns, containing a single 1,500-bp open reading frame encoding 499 amino acids including a signal peptide of 21 residues. ScAmy54 exhibited high amino acid homology with other liquefying fungal α-amylases essentially in the four conserved regions and in the putative catalytic triad. A 3-D structure model of ScAmy54 and ScAmy43 was built using the 3-D structure of 2guy from *A. niger* as template. ScAmy54 is composed by three domains A, B, and C, including the well-known $(\beta/\alpha)_8$ barrel motif in domain A, have a typical structure of α-amylase family, whereas ScAmy43 contained only tow domains A and B is the first fungal α-amylase described until now with the smallest catalytic domain.

*Corresponding author
Phone: +216-71-703-829, 216-71-703-627; Fax: +216-71-704-329;
E-mail: mnmarzouki@yahoo.fr

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α-Amylases (α-1,4-D-glucan-glucanohydrolase, E.C.3.2.1.1) which are widely distributed in animals, plants and microorganisms, catalyze the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and related polysaccharides. These enzymes have widespread applications in starch-processing, brewing, alcohol production, textile, and several other industries [56]. Although most α-amylases produce glucose or maltose as the major product from starch, amylases that specifically generate malto-oligosaccharides from starch have also been reported [1, 3, 41, 54, 60].

α-Amylases belong to the GH-13 family in the glycoside hydrolase classification and are composed by about 30 different enzyme specificities as hydrolases, transferases and isomerases [18, 28, 51]. These enzymes shared several common properties as the same number of highly conserved regions, the specific $(\beta/\alpha)_8$-barrel motif characteristic of the catalytic domain, the retaining mechanism of glycosidic bond hydrolysis and the presence within the catalytic site of the aspartate and glutamate served as nucleophile and proton donor respectively [18, 28].

X-ray diffraction studies of several α-amylases [20] have shown that they consist of three domains called A, B, and C [6]. The catalytic domain A contains an amino terminal $(\beta/\alpha)_8$-barrel structure, followed by a domain C formed by antiparallel β-strands. A third smaller domain (domain B) is present as a loop between the third β-strand and the third α-helix of the $(\beta/\alpha)_8$-barrel. In the same cases, α-amylase isolated from *Streptomyces limosus* contains an

extra domain (domain E) at their carboxyl-terminal region which is implicated in raw starch adsorption [26]. The central catalytic domain contains three critical invariant residues located at the bottom of the active site and whose substitution fully inactivates the enzyme [50]. Generally the α-amylases 3D structures contain a calcium ion with high affinity at a conserved calcium-binding site, which is located at the interface between domains A and B [29].

Carboxyl-terminal truncation has been described in α-amylases of *Bacillus subtilis* [38], *Pseudomonas stutzeri* [35] and barley [47], while artificial truncation has been performed on various amylolytic enzymes from *Bacillus subtilis* [31], *Bacillus stearothermophilus* [57], *Cryptococcus* sp. [15] and *Aspergillus kawachii* [21]. Although, most of the carboxyl-terminal-truncated enzyme retain the same level of amylolytic activity as the non-truncated enzyme [31, 35, 47], some truncations have been reported to affect the enzyme thermostability [31, 35, 57].

Sclerotinia sclerotiorum, a filamentous phytopathogen ascomycetes, is among the most nonspecific omnivarious and successful plant pathogens [7]. During plant infection this fungus produces a large number of hydrolytic enzymes such as endo/exopolygalacturonase, pectinmethylesterase, xylanase [12] and α-amylase [1, 2]. However, *S. sclerotiorum* has been poorly characterized at the molecular level and only few hydrolytic enzymes encoding genes have been characterized [40].

In the present study, we investigated the encoding gene sequence of a native and truncated α-amylases produced by *S. sclerotiorum*. We also described the enzyme properties based on sequence alignment, homology modeling and structural role of amino acid residues involved in binding and/or catalysis of the Ca^{2+} interaction. This study provides the first information on the structure and sequence of amylase-encoding gene from *S. sclerotiorum* strain and describes an active enzyme with minimal structural domains.

MATERIALS AND METHODS

Chemicals, Reagents, and Enzymes

Taq DNA Polymerase was from Qiagen, Oligonucleotides were from Biomers (France), IPTG, X-Gal, Trizol, DTT, and ampiciline were from Sigma, soluble starch was from Merck.

Culture Media and Growth Conditions

The phytopathogen *Sclerotinia sclerotiorum* was obtained from the national fungi collection of the cryptogamy laboratory (INRAT, Tunis). For enzyme induction and production, the fungus was grown in a minimal liquid medium supplemented with 1% (w/v) of soluble starch [30] and containing KCl, 1 gl^{-1}; $MgSO_4$, 0.5 gl^{-1}; KH_2PO_4, 1 gl^{-1}; $NaNO_3$, 1.4 gl^{-1}; $(NH_4)_2SO_4$, 4 gl^{-1}; yeast extract, 2 gl^{-1} and 1 mll^{-1} trace elements [1, 2]. pH was adjusted to 5.5 with HCl (1 N) before autoclaving. Cultures were then inoculated with mycelia disks cut from 3-days-old colonies and incubated at 25°C with orbital shaking (G24 Environmental incubator shaker-New Brunswick Scientific) at 150 rpm for 5 days.

The media for bacterial growth were LB (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) and LBA (LB containing 50 μg ampiciline/ml).

Amylase Activity Assay

α-amylase activity was measured by incubating the enzyme mixture (0.1 ml) with 1% (w/v) of soluble starch (0.5 ml) in 25 mM sodium acetate buffer, pH 4 at 37°C for 30 min. Reducing sugar was determined by the dinitrosalicylic acid method [34] and absorbance measured at 540 nm with D-glucose as standard. One unit of α-amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars per min, expressed in glucose equivalent.

MALDI-TOF Mass Spectrometry Analysis

The stained purified ScAmy43 band was excised and washed with 50% acetonitrile in 50 mM ammonium bicarbonate, pH 8, then sliced into small pieces and partially dried under vacuum. The gel pieces were rehydrated and digested into 100 mM ammonium bicarbonate, pH 8, containing 5 μg/ml trypsin [46]. Digestion was carried out for 4 h at 37°C and stopped by addition of 60% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water. The peptide mixture (1 μl) was mixed with an equal volume of matrix solution (4 mg/ml α-cyano-4-hydroxy cinamic acid in 50% acetonitrile and 0.1% aqueous TFA) and deposited onto the MALDI target plate using a MALDI-Q-TOF Premier instrument (Waters, Manchester, U.K.). External calibration covering the m/z 729–3,959 mass range [16] 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.

In Silico Digestion

The *in silico* digests was performed by trypsin using the Peptident Proteomic Tools in the ExPASY Molecular Biology Servers (htpp://www.expasy.ch/).

Database Mining of *S. sclerotiorum* Genome and Analysis of Predicted Proteins

The full genome sequence of *S. sclerotiorum* strain "1980" (ATCC 18683) has been deposited at the NCBI Database and Broad Institute Database (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html) with project accession number ID15529 and was used for Database mining. The nucleotide accession numbers of hypothetically *S. sclerotiorum* amylase genes are listed in Table 1. Hidden Markov Model (HMM) profiles were built with the HMMER package (http://hmmer.wstl.edu/) based on amino acid sequence of known member of GH13 [5]. Proteins belonging to this family were retrieved from the CAZY website at (http://www.cazy.org/) [11], and protein sequences were collected from the GenBank/Gen Pept Database at (http://www.ncbi.nlm.nih.gov/entrez/) and Swiss-Prot Database at (http://www.expasy.org/sprot/).

RNA Isolation and cDNA Synthesis

RNA was extracted from a *S. sclerotiorum* culture in the presence of 1% (w/v) starch using the TRIZOL method. Cells were suspended in 1 ml of TRIZOL reagent (Sigma) and 200 μl chloroform. The mixture was centrifuged (7,000 ×*g*, 30 min, 4°C) and RNA were

Table 1. All members of amylase family identified in the genome sequence of *S. sclerotiorum* "1980" using HMM and BLAST profiles.

Accession No.	Locus	Gene Name	Location	Length	Relevance	Matching Fields
XP_001598163	SS1G_00249	alpha-amylase precursor	Supercontig 1: 641703–643394–	1,692	1.76	blastx, hmmer
XP_001597582	**SS1G_01776**	**alpha-amylase-1-precursor**	**Supercontig 2: 1832833–1835000+**	**2,168**	**0.69**	**blastx**
XP_001595294	SS1G_03383	cell wall alpha-1,3-glucan synthase ags1	Supercontig 4: 774749–782298–	7,550	1.61	blastx
XP_001595296	SS1G_03385	alpha-amylase precursor	Supercontig 4: 786387–788463+	2,077	1.61	blastx
XP_001591169	SS1G_07794	1,4-alpha-glucan-branching enzyme	Supercontig 10: 736431–738783–	2,353	1.61	blastx
XP_001589670	SS1G_09392	hypothetical protein similar to starch binding domain-containing protein	Supercontig 13: 651303–652544+	1,242	0.17	blastx
XP_001588554	SS1G_10101	glucan 1,4-alpha-maltohexaosidase precursor	Supercontig 15: 120886–122621+	1,736	0.53	blastx
XP_001588435	SS1G_10882	alpha-amylase precursor	Supercontig 16: 890176–891971+	1,796	1.79	blastx, hmmer
XP_001588171	SS1G_10617	glucoamylase precursor	Supercontig 16: 221072–223364+	2,293	0.15	blastx
XP_001587859	SS1G_11100	alpha-glucosidase	Supercontig 17: 394018–395828–	1,811	1.15	hmmer
XP_001585588	SS1G_13472	alpha-amylase A type-3 precursor	Supercontig 25: 261484–263300–	1,817	1.99	blastx, hmmer

precipitated in the presence of 1/10 volume of 3 M sodium acetate and 3 volumes of isopropanol. Poly(A)$^+$ RNA were isolated from the solution with Quick prep micro mRNA purification kit.

Scamy54 cDNA was synthesized from mRNA by the reverse transcription procedure with a cDNA synthesis kit (Promega) using oligo (dT) as primer. Reverse transcription was carried out in a total reaction volume of 20 µl containing: mRNAs (5 µg) as template, 200U M-MLV reverse transcriptase (Invitrogen), 20 pmol of each deoxynucleoside triphosphate and 20 pmol of oligo dT. The cDNA/RNA heteroduplex was then denatured at 70°C for 15 min and immediately cooled on ice. Amplification of Scamy54 cDNA was carried out by PCR using the single strand cDNAs as template with specific primers.

Primer Design Polymerase Chain Reaction and Sequencing

S. sclerotiorum total DNA was prepared according to Sambrook et *al.* [43] with some modifications. Two overlapping fragments of α-amylase gene were amplified with polymerase chain reaction (PCR) using total DNA and cDNA as templates. The primers used were: for 2Scamy54F 5'TCGAATCAGGAGAAGTTCTTTCC3' and for 2Scamy54R primer 5'CCCAAGAATACCCTATTTCACCTC3' situated upstream and downstream of the gene and 1Scamy54F primer 5'GCAGCAGGTTGGAGATCACAATC3' and 1Scamy54R primer 5'TGTTGCAGATTGCGAGTAGGAGTT3' corresponding to the N-terminal amino acid sequence and the sequence EALWSNSNSYSQSATYYR previously determined from ScAmy43 by LC MS/MS [1]. PCRs were performed as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min and final extension step at 72°C for 5 min. PCR products for sequencing were recovered from the agarose (1% w/v) and purified using a DNA extraction kit (Qiagen). Nucleotide sequencing of the amylase gene fragments was performed using automatic sequences (Genome Express, France) based on didoxy chain termination method [44].

Cloning of Amplified PCR Product

Amplified PCR product of 1.7 Kb was cloned into PCR 2.1 TOPO TA-Cloning vector (Invitrogen). Recombinant colonies were obtained by transformation of Top10F' *E. coli* (Invitrogen) by the ligated mixture. Colonies were grown on nutriment agar plates with Ampicilline (100 µg/ml), X-Gal (100 mM) and IPTG (400 mM) for recombinants detection. Recombinant plasmid DNA was isolated from the selected white positive colonies by PCR control with specific primers.

Sequences Analysis and Deduced Amino Acid primary Sequence

DNA sequences were analyzed on automated DNA sequencer (Model 370A, Applied Bio systems). The cDNA nucleotide sequence was translated into protein and the deduced amino acid sequence was analyzed by the Expasy Swiss Prot Web Server (http://www.expasy.ch) [4]. Homology and identity analysis were carried out using BLAST through the NCBI server. The multiple sequence alignment was performed with the ClustalW program [55].

Computer Aide Modeling of *Sclerotinia* Amylase Tertiary Structure

The automated protein structure homology-modeling server, SWISS-MODEL [45] (http://www.expasy.org/swissmod/) was used to generate the three dimensional model. The Deep view Swiss-PDB viewer software from the EXPASY server (available at http://www.expasy.org/spdbv/) was applied to visualize and analyze the atomic structure of the model. Molecular modeling of ScAmy54 and ScAmy43 was resolved based on the X-ray crystallographic structure of *Aspergillus niger* α-amylase (reference PDB: 2guy) [58].

Nucleotide Sequence Accession Number

The nucleotide sequence data of the ScAmy54 gene was deposited in the GenBank Database under accession No. FJ773284.

RESULTS AND DISCUSSION

In a previously work [1, 2], we have biochemically studied two α-amylolytic enzymes produced by *S. sclerotiorum*, designated by ScAmy54 and ScAmy43 and having respectively a molecular weight of 54 and 43 kDa. So, we choose to investigate their structural and molecular properties by cloning and encoding genes analysis.

Identification of Scamy54 Gene in *S. sclerotiorum* "1980" (ATCC18683) Genome Bank

To identify genes encoding amylase enzymes, the *S. sclerotiorum* genome was screened with HMM and BLAST profile based on known enzymes from GH-13 family. The results lead to a retrieval a total of 11 previously unknown and hypothetically amylases proteins as listed in table 1. The predicted proteins were annotated based on their similarity to known enzymes and gene names were assigned based on this annotation.

In a previous work, ScAmy43 N-terminal and three internal amino acid sequences were determined by Edman degradation and *de novo* sequencing [1]. Comparison of sequenced peptides with sequence in the *S. sclerotiorum* genome Database led to the identification of a unique α-amylase gene under the accession number XP_001597582. The calculated molecular weight of this hypothetical amylase was 54 kDa corresponding to the ScAmy54 protein [2]. This result led us to think about the structural relationship between ScAmy54 and ScAmy43. Could they be two distinct isoforms? Or is ScAmy43 a proteolysis form of ScAmy54? To respond to these questions, we have made molecular and comparative studies.

Nucleotide Sequence Analysis

In order to study the structural relationship between ScAmy43 and ScAmy54, a molecular work was undertaken. Based on the nucleotide sequence published by Broad institute at SS1G_01776 locus and Supercontig 2: 1832833-1835000, oligonucleotides PCR were designed and synthesized.

A 2,300-bp and 1,700-bp fragments containing the α-amylase gene were amplified by PCR with *S. sclerotiorum* genomic DNA and cDNA using 2ScAmy54F and 2ScAmy54R as primers. PCR products were then directly sequenced to identify and determine the exon/intron junction of the α-amylase gene.

The nucleotide as well as deduced amino acid sequence of the Scamy54 gene product are shown in Fig. 1 and 2. The nucleotide sequence of the cDNA fragment reveals a single open reading frame (ORF), which begins with ATG codon at nucleotide 1 and ends with a TGA codon at nucleotide 1497. No other initiation codon was identified upstream the gene.

The open reading frame (ORF) consists of 1,500-bp encoding 499 amino acids corresponding to 54 kDa, It contains 8 introns exhibiting classical characteristics of filamentous fungal intron with GT and AG at the exon/intron junction [13].

The 12 amino acids (LSAAGWRSQSIY) characteristic of the N-terminal sequence previously determined by Edman degradation of the purified ScAmy43 [1] were localized at position 22–34. Consequently, a signal peptide of 21 amino acid residues long (MKPSSLLRLIPVT YLATSVAA) was present in the native sequence and which is removed during the secretion process. The signal peptide exhibits typical properties with positive charges at the N-terminus and followed by hydrophobic stretch (Fig. 2). Furthermore, its hydropathy index determined by Kyte and Doolittle method was 2.4 [24].

Amino Acid Sequence of ScAmy54 Analysis

The ScAmy54 amino acid sequence consists of a single polypeptide chain with approximately 25% α-helix and 25% β-sheet. This sequence exhibited a high degree homology to *Fusicoccum* sp. BCC4124 (60% identity and 76% similarity) [10], *Lipomyces kononenkoae* (60% identity and 75% similarity) [48] and *Aspergillus niger* (58% identity and 72% similarity) [21]. Homology was essentially found in the catalytic domain of α-amylases and the consensus amino acid residues were not found within the C and N-terminal sequence as for all fungal α-amylases activities. These results suggest that ScAmy54 have a separate domain structurally comparable with other fungal α-amylases.

Four conserved regions (I to IV) which are required for α-amylase catalytic activity [36] and characteristic of the α-amylase family are well conserved in ScAmy54 as DVVTNH, SIDGLRVD, EIFNGDP, and FLENHDVA for I, II, III, and IV regions respectively (Fig. 2). They are believed to form the active center, the substrate binding site and the calcium binding site. Furthermore, the seven amino acid residues which are highly conserved in the four conserved regions of the α-amylase family are completely preserved in ScAmy54 as well (Table 2).

The Scamy54 encoding sequence was compared to α-amylase-1-precursor (SS1G_01776-XP_001597582) deposited at the NCBI Database. It appeared longer by 150-bp corresponding to 50 amino acids distributed on two peptides of different size, the first IWISPIVQNIVGDSV was located at position 78–93 and the second peptide LHARGMYLMVDVVTNHM GYLGCGTCVDYSIYTPFNS at position 126–161 (Fig. 2). This result suggests that some sequences are part of introns in the α-amylase-1-precursor

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   1  TCGAATCAGGAGAAGTTCTTTCCTTCATTGATCTCAATCCCCTCGCCCTCTTCCGGTGTC
  61  TTTTACTTCATATTGTATCCCTCACGGAACTCGATTCAAAATGAAGCCAAGCAGTCTTCT
 121  CCGTCTCATACCCGTCACGTACCTGGCGACTAGTGTCGCGGCTCTCTCAGCAGCAGGTTG
 181  GAGATCACAATCTATCTATCAAGTCATCACAGATCGTTTTGCACGTACCGATGGCTCTAC
 241  TACTGCTTCTTGTAACGTGAATGAATATTGTGGTGGTTCGTGGCAGGGTATTATCAAACA
 301  TTTGGATTATATTCAGAATATGGGTTTCACGGCTGTGAGTTTGGAGATGTGGATTGGATG
 361  ATGGGTTTTGTGCTGATGATGAGTAGATTTGGATTTCTCCGATTGTGCAGAATATTGTTG
 421  GGGATAGTGTGGTATGCAAGTCTTTTTCGTTTTCCTCTTTGGAGAGAAGGGTTCTATATT
 481  TTCGGATAGACTATCTACGAGTGTCTGAACTGATGTGAAATTATAGGATGGTTCTAGTTA
 541  TCATGGATATTGGGCACAGAATATTTACCAGGTTAATTCCAATTTCGGTACACCAGCCGA
 601  TCTTAAAGCACTATCAGCTGCAGTAAGTAATCCAAGTCTACAACCAAAAGACGTAGTCAG
 661  CACTAACCTTATTCTGCACAGCTCCATGCTAGAGGCATGTACCTAATGGTCGACGTCGTC
 721  ACCAACCACATGGGATATCTCGGCTGCGGAACATGCGTAGACTATAGTATCTACACACCC
 781  TTCAATTCCGTGGGTGTTCTTCCTCACCAATAAACATGACATACATGACTAATCCACACG
 841  AATAGAAATCCTACTACCATCCTTTCTGCCTAATAGATTATAACAATGCAACAAGCGTGG
 901  TAGATTGTTGGGAAGGTGACAATATCGTCTCATTACCCGATCTTAGAACCGAAGATTCAG
 961  ATGTTTACACGGAGTGGAATTCGTGGATTTCACAACTCGTGGCTAATTATTCGATTGATG
1021  GGTTGAGAGTTGATAGTGCGCAACAAACTGGGAAGGCTTTCTTTCCTAGTTTTCAGAATT
1081  CGGGTATGTGGTTTTTTTCAAGATTTTTTTTGTGCTCGGTAATGGGGACATGGAGATAGA
1141  ACAAAGAGGCTAACAATGTATTACATAGCTGGTGTATACGTCGTTGGAGAAATTTTCAAT
1201  GGTGATCCAGCCTACGTATGTCCATACCAGAATTACATGAACGGAGTTCTCAACTATCCC
1261  GCGTAAGTCCTGTTTCTCATCACTAAGCAACCCCGCAAAACTCTTCCCGATCGTAAAACT
1321  AAATTAAGTAGATACTACTGGATAACACAAGCCTTCCAATCCACAAGCGGAAGTATCTCC
1381  AACCTCGTAAATGGAATCAACACGATGAAATCCAGCTGTTCAGATACCACTCTCCTTGGA
1441  TCCTTCCTAGAAAATCACGACGTGGCTCGTTTCCCCTCTTATACATCCGATGCCTCACTC
1501  ACTAAAAATGCCATCGCATTTACCATTCTCTCAGATGGCATTCCCATCGGTAACTCCCCC
1561  TCTCCCTTCCCCATTCCTTCCCCCTCCCCCAACATCCCAAAATCCAACTAACCCTCCTCC
1621  CAAACCCCAGTCTACCAAGGCCAAGAACAACACCTAACCGGTTCCTCCGTCCCCAACAAC
1681  CGCGAAGCCCTCTGGTCCAACTCAAACTCCTACTCGCAATCTGCAACATATTACCGCTTT
1741  ATTGCTTCGGTGAATCAAATTCGTAATCAGGCTATTTACGTGGATCCGACCTATCTTACT
1801  TATAAAGCATATCCGGTTTATAGTGATGGTACGACGATTGTGATGAGGAAGGGTTTTACC
1861  GGAAAGCAGATTATTGCTGTTTTTAGTAATAAGGGTGCTTCGGGGAGTAGTGAGTTTATT
1921  TTTTATTTTTATTTTTTTTTGCTTATATTTCCTAAATCTTAAATTTTAACTTTCAACCC
1981  ACTTGAATATTTTCCTCAAAAAATTCTCGAGGAAATGTCTTTGGGATTGGGGAAAGAAAAG
2041  ATCTTCCACGGAAATAATCGCTAACAAATCCCACCCACAGGTTACACCTTAACACTAACA
2101  AGTTCACAAACGGGATTCACGTCTAATCTGCAAGTGGTGGAAGTCCTTACGTGCACGACG
2161  TCTACGACGAATGGTAGTGGGAATTTGGCGGTTAGTATGGCGGGGGGTGTGCCGAGGATC
2221  TTTTATCCAAAGTCGTATTTGGTTGGGAGTGGGGTTTGTTCTTTGTGAGTGGGAGTTTTT
2281  ATAGATGGATGGATGGATGGATGGATAGAGATGTATAAGGTGAGGTGAGATGAGATGAGG
2341  TGAATAGGGTATTCTTGGG
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Fig. 1. Nucleotide sequence of the Scamy54 gene.
The dotted line indicates the internal consensus sequence.

sequence (SS1G_01776-XP_ 001597582), although we demonstrate that the two sequences constitute a real part of the coding sequence of the ScAmy54. We further specified the coding sequence of the uncompleted amylase sequence from NCBI Database. The first highly conserved region of ScAmy54 is not found in the sequence of α-amylase-1-precursor (SS1G_01776-XP_ 001597582). This region contains highly conserved residues, located in the C-

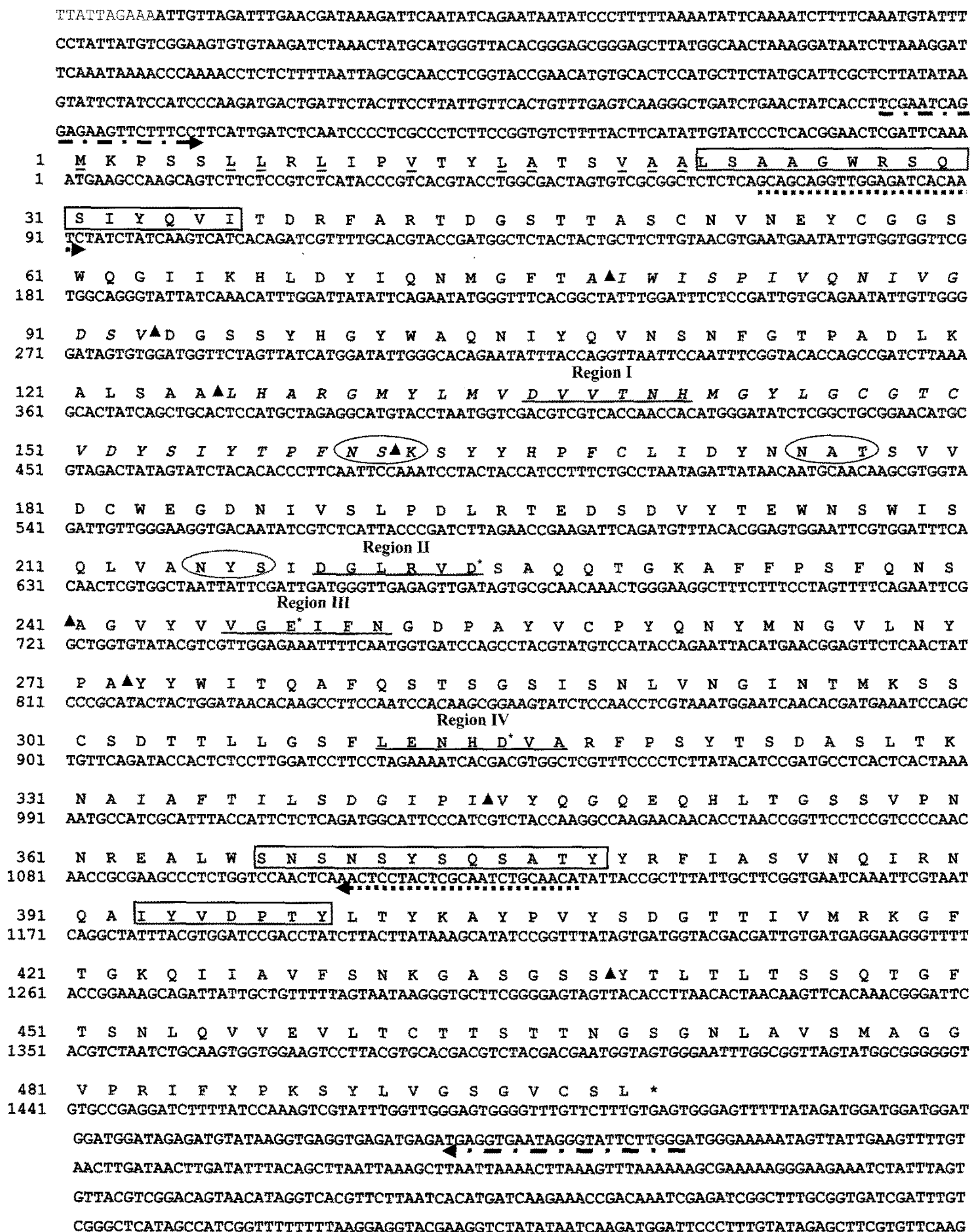

Fig. 2. Nucleotide sequence of Scamy54 cDNA and the deduced aminoacide sequence and their flanking regions.
The nucleotide sequence numbered from the first base initiation codon. asterisk (*) indicates a stop codon. The N-terminal and internal amino acid sequence of ScAmy54 are boxed. Bold lines indicate hydrophobic amino acid residues in the signal peptide. Solid arrowheads indicate the localization of intron sequences. Region I, II, III, and IV indicate conserved regions of α-amylase. Circular boxes indicate the site possible to combine with sugar chain. Italic peptides correspond to those specific of ScAmy54 and don't found in the α-amylase-1-precursor (SS1G_01776-XP_ 001597582) sequence deposit in the NCBI Database. ·······▸ and –·–▸ indicate the annealing position of used primers.

terminal part of the third β-strand (β3) and which are implicated in the integrity of the active site and in the conformational stability of enzyme as well as in the coordination of calcium ion between domain A and B [6, 37].

Identification of the Proteolysis Site in ScAmy54

The molecular weight of protein estimated from nucleotides sequence was 54 kDa, whereas purified enzymes as determined by SDS–PAGE had a molecular weight of 43 kDa [1] and 54 kDa [2]. Comparative studies between

Table 2. Comparison of amino acid sequence four regions conserved among ScAmy54 of *S. sclerotiorum* and various fungal α-amylases.

Source of α-amylase	Region I	Region II	Region III	Region IV
S. sclerotiorum	DVVTNH	SIDGIRVD	EIFNGDP	FLENHDVA
Fusicoccum BCC4124	DVVVNH	SIDGIRID	EVYDGDP	FSENHDIT
Neo. fischeri NRRL	DVVANH	SIDGIRID	EVFSGDP	FIENHDNP
Penici. marneffei	DVVANH	SIDGIRLD	EVFNGDA	FSENHDNP
Emir. nidulans	DVVANH	SIDGIRID	EVFQGDP	FIENHDNP
Chizosac. pombe	DIVVNH	QFDGIRLD	EVFHGDP	FLENHDFP
A. kawachii	DVVANH	SIDGIRID	EVLDGDP	FVENHDNP
A. shirou	DVVANH	SIDGIRID	EVLDVDP	FVENHDNP
A. awamori I	DVVANH	SIDGIRID	EVLDGDP	FVENHDNP
A. awamori III	DVVPNH	SVDGIRID	EVDNGNP	FIENHDNP
A. awamori A	DVVANH	SIDGIRID	EVLDGDP	FVENHDNP
A. awamori B	DVVANH	SIDGIRID	EVLDGDP	FVENHDNP
A. flav	DVVANH	SIDGIRID	EVLDGDP	FVENHDNP
A. oryzae	DVVANH	SIDGIRID	EVLDGDP	FVENHDNP
A. fumigatus	DVVANH	SIDGIRID	EVFSGDP	FIENHDNP
A. niger	DVVPNH	SVDGIRID	EVDNGNP	FIENHDNP
		*	*	*

Region I, II, III, and IV are the four well-known conserved sequence regions within the α-amylase family of enzymes. Shadowed boxes indicate the seven amino acid residues of ScAmy54 which are strictly conserved in the four regions of the α-amylase family. The three proposed catalytic residues are indicated with (*).

in situ and *in silico* tryptic digest of ScAmy43 and ScAmy54 were conducted to explain these discrepancies. MALDI mass spectra of tryptic digest of ScAmy43 showed all *de novo* sequenced peptides SQSIYQVITDR, NQAIYVDPTY LTYK and EALWSNSNSYSQSATYYR into the intense peaks at m/z 1309.6768, m/z 1688.8608 and m/z 2126.9558 respectively and they corresponds to theoretical masses of tryptic peptides derived from the *in silico* analysis of ScAmy54 polypeptide sequence (Fig. 3). In agreement with MALDI mass spectra two peptides AYPV YSDGTTIVMRKGFTGK and QIIAVFSNK that should correspond to the two peptides peaks at m/z 2191.1216 and m/z 1019.5884 respectively are absent. This result conducted us to predict with a high probability value the cleavage site location between the two peptides NQAIYVD PTYLTYK and AYPV YSDGTTIVMRKGFTGK. Although this analysis indicates clearly that the C-terminal truncation could be achieved from the peptide sequence NQAIYVDPTYLTYK and we can conclude that ScAmy43 might be a proteolytically processed C-terminal fragment of the native ScAmy54 α-amylase.

The result of proteomic and genomic analysis suggested that the two enzymes originated from the same α-amylase gene and that truncation of ScAmy54 to ScAmy43 might be occurred probably during the cultivation of *S. sclerotiorum.* The multiplicity of isoforms was also observed in various microorganisms such as in the fungus *Aspergillus awamori* [32] and in the alkaliphilic *Bacillus* sp. Gm 8901[23]. Furthermore, three α-amylolytic isoforms of the alkaliphilic *Bacillus* sp.H-167 were produced from a native enzyme by C-terminus proteolytically process [14]. Similar C-terminal domain truncation was observed in α-amylases of *Bacillus subtilis* [38], *Pseudomonas stutzeri* [35] and barley [47]. Experiments of proteolysis done on several amylases from various origins in particular of *B. subtilis* [31], *B. stearothermophilus* [57], *Cryptococcus* sp. [15], and *A. kawachii* [21] showed that some truncated amylases in there C-terminal sequence can maintained the same activity [35, 38, 47]. The ScAmy43 α-amylase with only catalytic domain, still remain active and constitute a smallest fungal α-amylase. However, no report of an active α-amylase in which more than 25% of the carboxyl-terminal polypeptide has been truncated. Indeed, the presence of the four conserved regions where the catalytic residues are essential for the preservation of the activity. The loss of one of these elements is reflected on the organization of the 3D structure and generates into an irreversible inactivation of the enzyme [59].

Tertiary Structure of ScAmy54 and ScAmy43

The 3-D structure of *Aspergillus niger* amylase (2guy) was used as a starting template to built models for ScAmy54, using the automated structure-modeling program Deep View/Swiss-PDB viewer. A representation of the overall polypeptide chain fold determined for ScAmy54 is shown in Fig. 4. This protein is composed of three structural domains: A largest domain A, which consists of an eight-stranded parallel β-barrel surrounded by a concentric cylinder of α-helical segments is constructed by two segments of polypeptides chain including residues 1–139

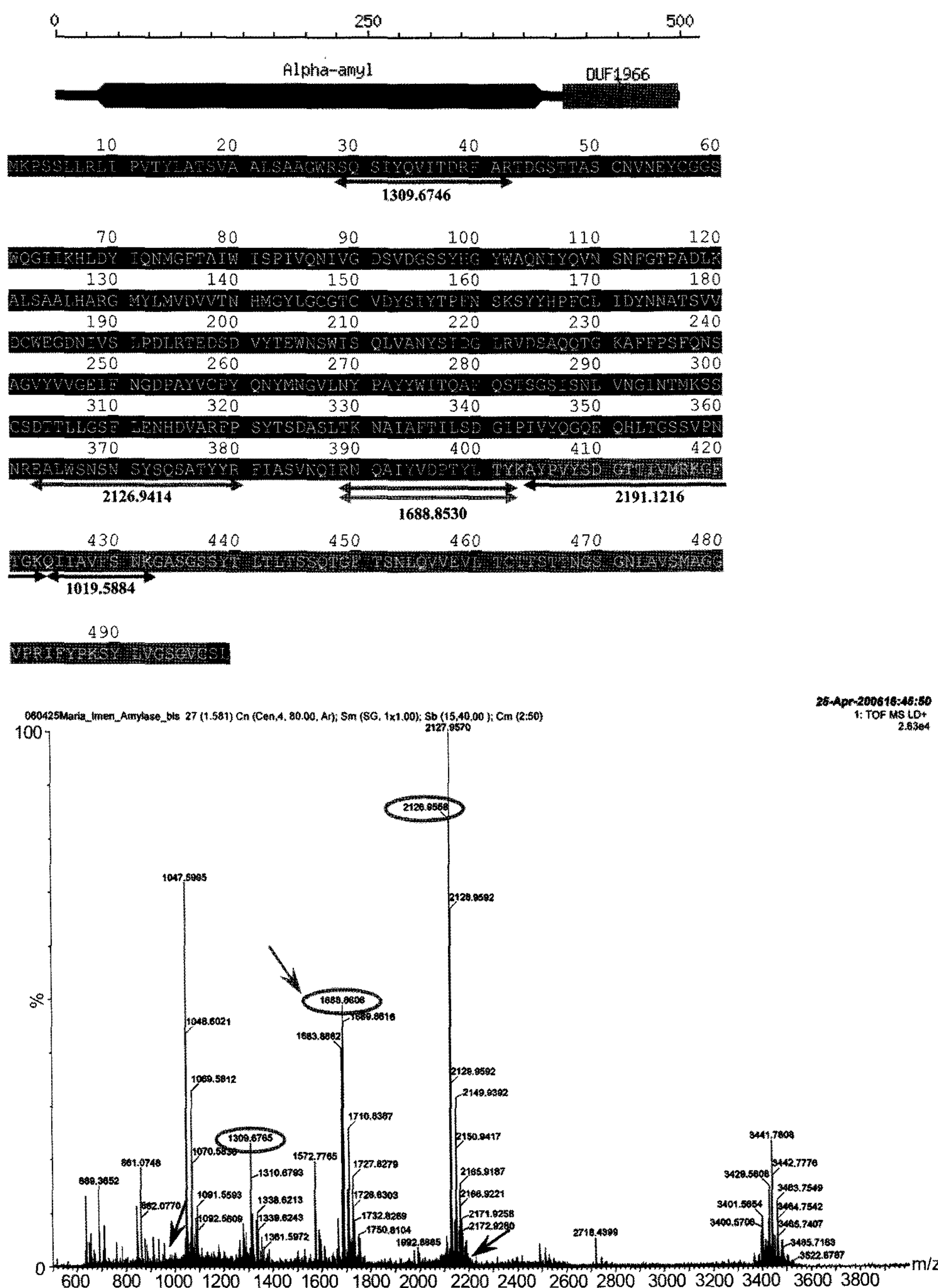

Fig. 3. Determination of the sequence cleavage site of ScAmy54.

Comparative study of the peptide mass fingerprint of the purified ScAmy43 and the peptide mass data obtained by *in silico* trypsin digestion of ScAmy54 predicted amino acid sequence. The two peptides AYPVYSDGTTIVMRKGFTGK and QIIAVFSNK that must correspond to the two peptides peaks at m/z 2191.1216 and m/z 1019.5884 respectively (bleu arrow) are absent and a cleavage site was situated between the two peptides NQAIYVDPTYLTYK (Orange arrow) and AYPVYSDGTTIVMRKGFTGK with a high probability value. Encircled m/z peaks correspond to the previously sequenced peptides by nano LC MS/MS [1].

and 199–399. The domains B is build from residues 140–198, occur between the third β-strand and α-helix of the central β-barrel of domain A. The domain B has an irregular β-rich structure, and varies substantially in size and structure among α-amylases [19]. This domain had an important role in the substrate binding and specificity differences observed between α-amylases [28]. The ScAmy54 domain C constitutes the C-terminal part of the sequence from residues 400 to 499. This domain folds in a β-barrel structure containing Greek key motif and is loosely associated with domain A (Fig. 4). In terms of primary sequence, the domain C constitutes the most variable

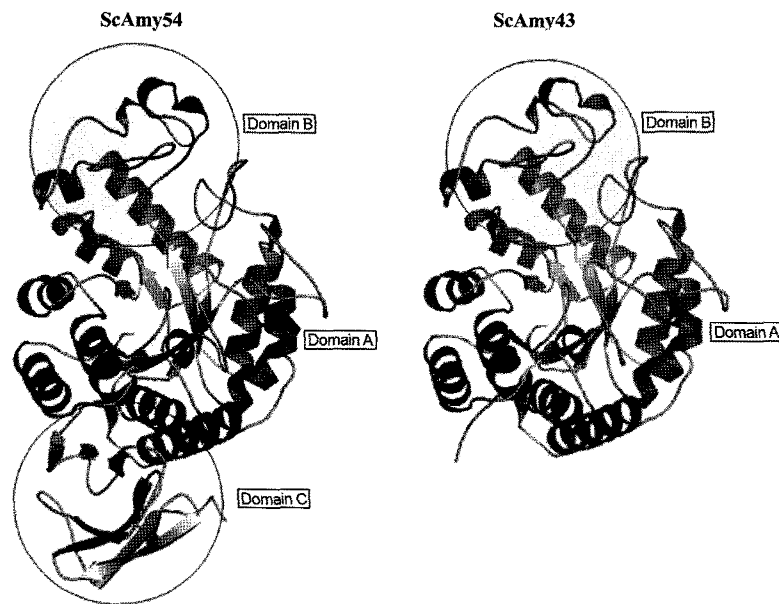

Fig. 4. Stereo view of the ribbon model of the overall structure of ScAmy 54 and ScAmy43.
ScAmy54 contain all the three domains (A, B, and C) characteristic of the α-amylase family. Domain A, the most well conserved $(\beta/\alpha)_8$-barrel domain in α-amylases, is shown in the middle (helices in red, β-sheets in yellow and random coil in green). Domain C, which is composed of β-strands with the so-called Greek key motif is absent in ScAmy43.

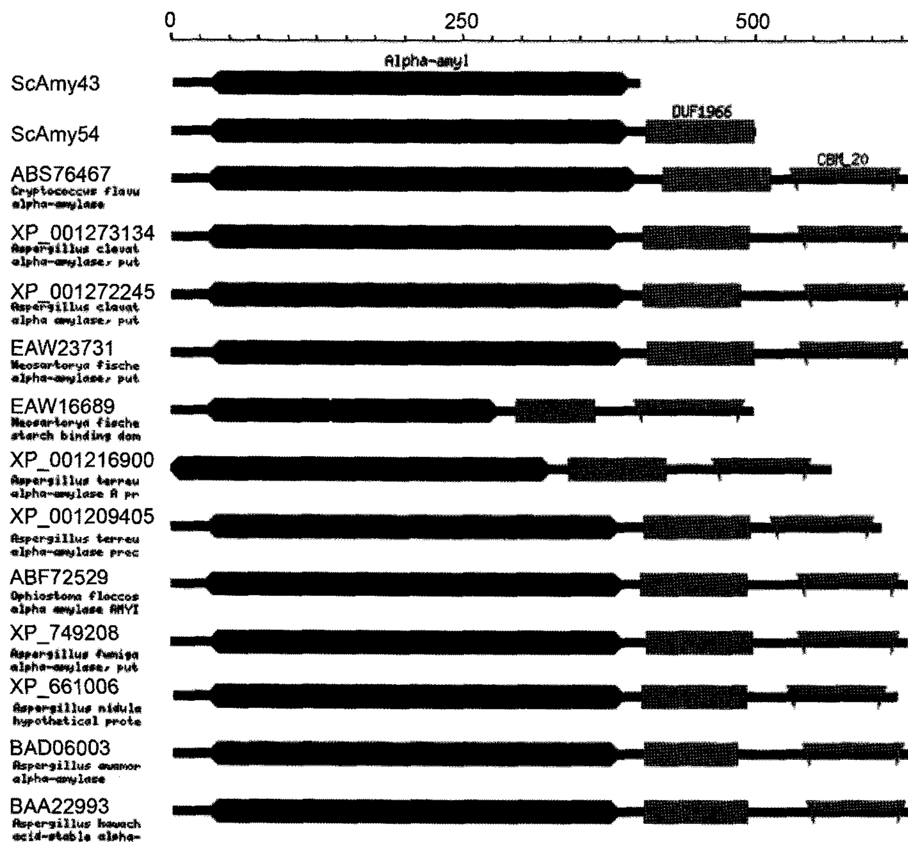

Fig. 5. Schematic representation of the α-amylase family domain organization as proposed by Jespersen et *al.*, 1991 [20].
The blue areas indicate the catalytic domain. Red areas indicate the DUF1966 domain corresponding to the domain C that it is functionally unknown but clearly identified by sequence comparison and hydrophobic-cluster analysis. Orange areas indicate the CBM_20 domain corresponding to the starch binding domain.

domain between α-amylases of different origins [28, 42] and its exact function has not been yet defined [8]. However, ScAmy43 has the same N-terminal domain organization of ScAmy54 (domain A and B) but lacks the 96 amino acids of the domain C (DUF1966 domain) (Fig. 5). The difference between the two enzyme forms is intriguing and could correspond to a structural truncation. This modification has no effect on the biochemical properties of ScAmy43 compared to those of ScAmy54. In fact all structural and functional data obtained to date support the conclusion that only the whole of the $(\beta/\alpha)_8$-barrel is essential for amylase activity and that the 96 carboxyl-terminal amino acids were not necessary for the functionality and for the folding of ScAmy43.

S. sclerotiorum proteases could attack native ScAmy54 to give ScAmy43 as reported for the α-amylase-pullulanase from *Bacillus* sp. Strain XAL601 [53]. Ke *et al.* [22] discussed the smallest part of the catalytic domain with that an α-amylase which conserved its function and proved that the $(\beta/\alpha)_4$-half-barrel is the minimal subdomain necessary to maintain amylase function identified to date.

As shown in Fig. 5, ScAmy54 doesn't contain the predicted Starch Binding Domain (SBD), whereas its homologues in *A. nidulans*, *A. funigatus* and other *Aspergillus* contain a full length SBD. This domain generally located in the C-terminal region has a raw starch affinity and is linked to domain C by a TS domain rich in Threonine and Serine. With similar structure characteristic ScAmy54 and ScAmy43 are unable to digest raw starch as confirmed by previous experimental results [1].

Active Site and Catalytic Amino Acids

The active site, located in the interface of domain A and B in ScAmy54, contains three essential catalytic residues Asp224, Glu248, and Asp315 (Fig. 6A). Asp224 and Glu248, are believed to participate in the catalytic mechanism of ScAmy54 as proton acceptor (nucleophile) and proton donor (acid) respectively [33]. The role of the second aspartic acid is less uncertain, but it has been suggested to be involved in stabilizing the oxycarbonium ion-like transition state and also in maintaining the glutamic acid in the correct state of protonation for activity [27].

After sequences alignments and mutagenesis studies, corresponding residues could be defined for other carbohydrate converting enzymes that share the same catalytic mechanism [17]. Mutation of these residues reduced activity and/or altered reaction specificity of the enzyme [25].

The Calcium-Binding Site

The calcium binding site is located between the C-terminus of the central β-barrel and domain B and serves as a link between the two domains. The architecture of the calcium binding site of ScAmy54, as depicted in Fig. 6B revealed four residues serve as ligands, three of them are strictly conserved: an asparagine (Asn121) in the loop region between domain A and B, an aspartic acid (Asp175) at the end of domain B, and the carbonyl oxygen atom of the histidine (His210) in the highly conserved region from the beginning of Aβ4 to the beginning of Aα4. The reason for the almost complete conservation of histidine residue may be due to its role in interaction with the fourth sugar unit of the saccharide substrate accommodated in the active site. The fourth residue (Glu162) is located in a region of domain B. Three water molecules complete the co-ordination sphere.

This common calcium site is strictly conserved among distantly related α-amylases from mammals [39], insects [49], plants, bacteria [29] and fungal [52] and can be regarded as a distorted pentagonal bipyramid as described by Boel et *al.* [6] for *Aspergillus niger* α-amylase. Additional calcium ions have been found in *Bacillus* α-amylases [9].

It is the common feature of α-amylases that calcium ion is required for their structural integrity, thermal stability, as well as enzymatic activity. It has been shown that the

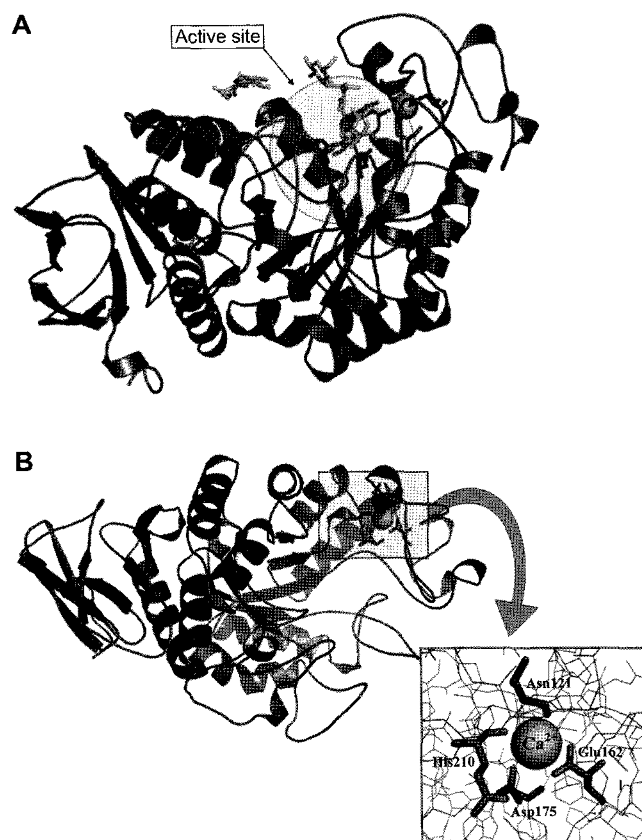

Fig. 6. Active and calcium binding sites in ScAmy54.
A. Stereo view of the active site region of ScAmy54 in complex with a bound oligosaccharide (green stick) showing catalytic residues (labeled in red) along with the remaining substrate-binding residues. **B.** Stereo views of Ca^{2+} binding sites of ScAmy54. Pentagonal bipyramidal cages surrounding metal ions are Asn121, Glu162, Asp175, and His210 and the calcium ion is represented by a green sphere.

removal of calcium upon adding chelators often leads to a reduction in thermostability and enzyme activity with respect to α-amylases [37]. Already, addition of 5 mM $CaCl_2$ has improved at 10% ScAmy54 activity and thermostability [2].

In conclusion and to our knowledge this is the first report of proteomic and molecular studies of α-amylase activities from a strain of the genus *S. sclerotiorum*. A 1.7-kb cDNA fragment containing the complete ScAmy54 gene from *S. sclerotiorum* was cloned and sequenced. The ScAmy54 ORF encodes a 499 amino acids polypeptide with a calculated molecular weight of 54 kDa. The deduced amino acid sequence of the α-amylase gene exhibited a high degree of homology to several fungal α-amylases especially in the catalytic domain. The difference in the molecular weight of the purified ScAmy43 and predicted ScAmy54 sequence could be explained by a domain truncation. This hypothesis was confirmed by a molecular and structural comparative study and we conclude that ScAmy54 and ScAmy43 were produced from the same gene and ScAmy43 arose from ScAmy54 by probably a processing of its carboxyl-terminal region during the *S. sclerotiorum* cultivation. The functional ScAmy43 described in this report, containing only the domain A and B, is the smallest subdomain of active fungal α-amylases identified to date. Expression of ScAmy54 and ScAmy43 gene from *S. sclerotiorum* will help us to elucidate the C-terminal domain role. Experimentation in this direction is now in progress.

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