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An. Acad. Bras. Ciênc., v.84 , n.2, p. 469-486, 2012
http://www.producao.usp.br/handle/BDPI/38028

Anais da Academia Brasileira de Ciências (2012) 84(2): 469-486
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
www.scielo.br/aabc

Purification, characterization and structural determination of chitinases produced by *Moniliophthora perniciosa*

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Manuscript received on November 29, 2010; accepted for publication on May 31, 2011

ABSTRACT

The enzyme chitinase from *Moniliophthora perniciosa* the causative agent of the witches' broom disease in *Theobroma cacao*, was partially purified with ammonium sulfate and filtration by Sephacryl S-200 using sodium phosphate as an extraction buffer. Response surface methodology (RSM) was used to determine the optimum pH and temperature conditions. Four different isoenzymes were obtained: ChitMp I, ChitMp II, ChitMp III and ChitMp IV. ChitMp I had an optimum temperature at 44-73°C and an optimum pH at 7.0-8.4. ChitMp II had an optimum temperature at 45-73°C and an optimum pH at 7.0-8.4. ChitMp III had an optimum temperature at 54-67°C and an optimum pH at 7.3-8.8. ChitMp IV had an optimum temperature at 60°C and an optimum pH at 7.0. For the computational biology, the primary sequence was determined in silico from the database of the Genome/Proteome Project of *M. perniciosa*, yielding a sequence with 564 bp and 188 amino acids that was used for the three-dimensional design in a comparative modeling methodology. The generated models were submitted to validation using Procheck 3.0 and ANOLEA. The model proposed for the chitinase was subjected to a dynamic analysis over a 1 ns interval, resulting in a model with 91.7% of the residues occupying favorable places on the Ramachandran plot and an RMS of 2.68.

Key words: Chitinase, *Moniliophthora perniciosa*, kinetic characterization, purification, isoenzymes, heat stability, 3D structure, comparative modeling.

**In memoriam*
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INTRODUCTION

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (Glc-NAc) residues linked by β-1,4 bonds, is a common constituent in cocoons, exoskeletons and peritrophic membranes of insects, crustacean shells, and fungal cell walls (Wang et al. 2002, Schrempf 2001, Nicol 1991, Gooday 1977). Also, all organisms that contain chitin have chitinases (EC 3.2.1.14) and other chitinolytic enzymes, as these enzymes are presumably required for the morphogenesis of cell walls and exoskeletons (Patil et al. 2000, Dahiya et al. 2006). Chitinases are glycosidases; they are also found in organisms that do not contain chitin, such as plants, bacteria, and viruses. Although the physiological roles of these enzymes remain unresolved, it is clear that plant enzymes are involved in the defense against fungal pathogens and in regulating development (Merzendorfer 2006, Dahiya et al. 2006, Duo-Chuan 2006). Filamentous fungi occupy a delicate balance between biosynthesis and hydrolysis of the cell wall during the remodeling for hyphal growth, hyphal branching and septum formation, thereby requiring the involvement of chitinolytic enzymes in this process (Dahiya et al. 2006, Selvaggini et al. 2004).

Endochitinases, exochitinases and beta-N-acetylhexosamidases are the main chitinolytic enzymes, and typically several hydrolytic enzymes act synergistically to accomplish the efficient and complete biodegradation of chitin (Dahiya et al. 2005, Bormann et al. 1999).

The production of microbial chitinases has received remarkable attention in both the industrial and scientific communities, mainly because of theirs enormous potential for economically increasing the production of several useful products (Dahiya et al. 2006, Felse and Panda 1999a, b, Kim et al. 2003). These enzymes have widespread applications in the fields of medicine (Kim et al. 2003, Toharisman et al. 2005), waste management (Odds 2003, Lopes et al. 2008), biotechnology (Felse and Panda 1999a, b) and industry (Haki and Rakshit 2003). Chitinases are used for the mild degradation of chitin to prepare biologically active chitooligosaccharides and for the structural determination of the glycoprotein polysaccharide. This application of chitinase requires either commercially available individual chitinases or at least a mixture of endochitinases and exochitinases free of other proteins (Tikhonov et al. 1998). In the agricultural field, chitinases are used mainly to control phytopathogenic fungi (Felse and Panda 1999a, b, Gobbetti et al. 1999). Chitinolytic enzymes from both bacteria (*Aeromonas* and *Serratia*) and fungi (*Gliocladium* and *Trichoderma*) have been used successfully for the control of pathogenic fungi of plants (Chernin et al. 1995). *Moniliophthra perniciosa*, the causative agent of the witches' broom disease in *Theobroma cacao*, is responsible for major crop losses in South American and Caribbean cocoa plantations (Kilaru and Hasenstein 2005, Scarpari et al. 2005, Surujdeo-Maharaj and Umaharan 2004). In 1989, witches' broom disease was identified in Bahia, the leading cocoa-growing region in Brazil (Purdy and Schmidt 1996, Griffith et al. 2003). In less than 10 years, the cocoa production in Brazil decreased from 400,000 tons to 100,000 tons (Hebbar 2007). Consequently, Brazil slipped from being the third- to the fifth-largest cocoa-producing country in the world (Pereira et al. 1990). The pathogen is a hemibiotrophic basidiomycete with two distinguishable phases in its life cycle.

The concept of response surface methodology (RSM) has eased the process of development and has been of significant industrial use. Recent studies have indicated that RSM can be used to analyze the effects of different factors on proteolytic activity (Gobbetti et al. 1999) and to optimize the enzyme production (Nawani and Kapadnis 2005, Tikhonov et al. 1998).

In this study, we describe the isolation, partial purification and biochemical characterization of the chitinase from *M. perniciosa*. The 3D structure of the protein was elucidated by comparative modeling.

MATERIALS AND METHODS

Materials

Chitin and bovine serum albumin were purchased from Sigma Chemical Co (St Louis, MO). All the other chemicals used were of high quality analytical grade.

The microorganism used in this study was *M. perniciosa* (CCMB0257) from the Collection of Cultures of Microorganisms of Bahia (CCMB).

Microorganism and Enzyme Production

M. perniciosa (CCMB0257) was maintained on potato dextrose agar plates at 25°C. To produce chitinase, *M. perniciosa* was grown in vegetative brooms cultivated in a special system with conditions amenable to fungi development (basidiocarps).

Fig. 1 - Scheme to produce *M. perniciosa*.

Extraction of the Chitinase and Ammonium Sulphate Fraction

The enzyme was extracted at 40°C with 0.05 M sodium phosphate buffer at pH 7.0 containing 0.6 M NaCl. The ratio of *M. perniciosa* to the extraction buffer was 1:3 (g mL^{-1}). The homogenate was squeezed through two layers of gauze and the extract was centrifuged at 10,000 x g for 10 min to remove the solid particles. The precipitate was discarded, and the supernatant was brought to 70% saturation by adding solid ammonium sulfate and centrifuged at 10,000 x g for 10 min after standing for 1 h. The precipitate with high PME activity was resuspended in borate acetate buffer at the ratio of 1:3 (w/v) and stored at low temperature.

The samples were desalted by ultra filtration with an Amicon membrane (10000 Da).

Measurement of Enzyme Activity

Chitinolytic activity was determined using the colloidal chitin as the substrate. A 0.2 ml portion of the enzyme extract was mixed with 0.2 ml of 1% chitin in 0.05 M phosphate buffer at pH 7.0.
The assay mixture was incubated at 50°C and the end products of the reaction were analyzed using the DNS method (Miller 1959). One unit of chitinase activity is defined as the amount of enzymes required to release 1 µmol of GlcNAc in 1 min under the above.

Protein Determination and Enzyme Analysis

Protein concentration was determined by the method of Bradford using bovine soroalbumin as a standard (Bradford 1976).

Chromatographic Separation

Sephacryl S-200 chromatography

Following an initial round of Sephacryl S-200 (20.0 x 1.1 cm) chromatography, fractions containing chitinase activity were pooled and applied to a

Sephacryl S-200 column (20.0 x 1.1 cm) previously equilibrated with ammonium sulfate buffer, pH 7.0 (the samples were subjected to a second round of chromatography). The same buffer was also used for elution, and 1.5 ml fractions were collected and assayed for chitinase activity.

Heat Stability

Chitinase samples in test tubes (selected to be equal in weight, volume and size) were incubated in water at different temperatures and for different times. After the heating process, the tubes were cooled in melting ice and the residual activity was measured. The residual activity was determined after incubation: 80°C (0, 10, 20, 30, 40, 50 and 60 min) and 90°C (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 min).

Experimental Design

A double-variable, three-level central composite design (CCD) leading to 11 sets of experiments (assays), performed in a triplicate, was used to verify the optimum pH and temperature optimum.
The STATISTICA 6.0 software (StatSoft, Tulsa, OK) was used to generate design matrix and analyze the results. Table II shows the design matrix and responses (results) obtained for the chitinase activity.

Replications and Statistical analysis

The extractions and chromatographic isolations were repeated at least three times. Significant differences among sample means were tested.

Primary Structure Determination

The sequence of the chitinase of the fungus that causes witches' broom disease was obtained by in silico analysis of reads (Fig 5, Table III) deposited in the Genome Project *M. perniciosa* and in assembly of a contig.

Chitinase Models

Chitinase models were built by threading using the Modeller program and four structures as templates and by homology comparison using the program Deep View v3.7 (Humphrey et al. 1996) and the models 1WNO and PDB 2A3E as templates (Table V), which were obtained from the PDB (Protein Data Bank) with 100% similarity with the sequence problem.

RESULTS

PURIFICATION OF CHITINASE

Chitinase was extracted from *M. perniciosa* as described in Materials and Methods. The purification scheme consisted of only three steps: ammonium sulfate precipitation, gel filtration on Sephacryl S-200 and re-chromatography in gel filtration on Sephacryl S-200.

Ammonium sulfate precipitation of the enzyme resulted in a 1.63-fold increase in a specific activity. Subsequently, chitinase was eluted on a Sephacryl S-200 column equilibrated with a 50 mM sodium phosphate buffer at pH 7.0. The samples were re-eluted on a Sephacryl S-200 column. The result of this step was a 18.16-fold purification for ChitMp I_2, a 7.83-fold purification for ChitMp II_2, a 16.67-fold purification for ChitMp III_2, and a 10.42-fold purification for ChitMp IV_2 (Figure 2 and Table I)

Effect of Temperature on Enzyme Activity

The thermal stability of the chitinase was studied by heating the samples at 80 and 90°C for various time periods. The residual activity was measured as described in Materials and Methods.

As shown in Figure 3a, ChitMpI_2 lost 10% of their initial activities after 20 min of heating at 80°C, and were inactivated completely after 60 min of heating. Furthermore, the enzyme lost 100% of their initial activities after only 2 min of heating at 90°C (Figure 3b).

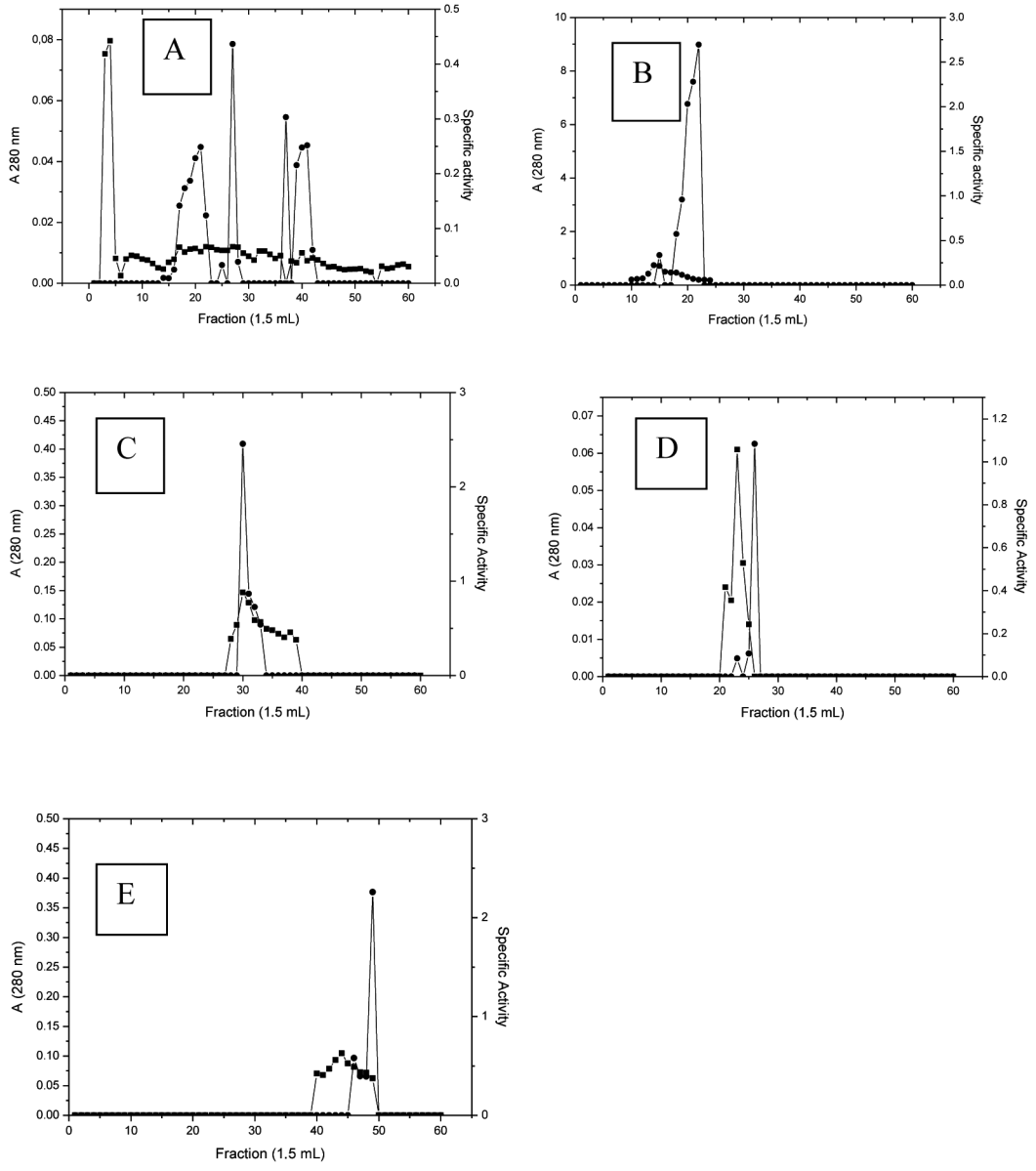

Fig. 2 - Purification of the chitinase from *M. perniciosa* by Sephacryl S-200 column chromatography: (●) chitinase activity; (■) protein content. **(A)** Crude extract, **(B)** ChitMp I, **(C)** ChitMp II, **(D)** ChitMp III, **(E)** ChitMp IV.

TABLE I
Extraction and purification of chitinase

Protein Step	Activity (Units)	Protein (mg/mL)	Specific Activity (units/mg protein)	Purification factor
Crude extract	0.018	0.15	0.12	
Ammonium sulfate precipitation	0.059	0.30	0.196	1.63
Sephacryl S-200 - ChitMp I_1	0.0082	0.0435	0.19	1.58
Sephacryl S-200 - ChitMp II_1	0.0134	0.039	0.34	2.83
Sephacryl S-200 - ChitMp III_1	0.0125	0.056	0.22	1.83
Sephacryl S-200 - ChitMp IV_1	0.0126	0.070	0.18	1.5
Sephacryl S-200 - ChitMp I_2	0.037	0.017	2.18	18.16
Sephacryl S-200 - ChitMp II_2	0.0177	0.0201	0.88	7.83
Sephacryl S-200 - ChitMp III_2	0.04	0.02	2.0	16.67
Sephacryl S-200 - ChitMp IV_2	0.03	0.024	1.25	10.42

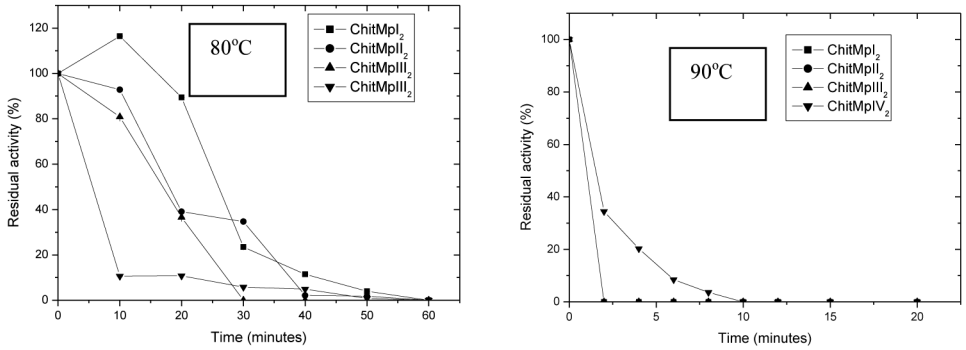

Fig. 3 - Heat stability of chitinase. All reactions were carried out at pH 7.0 with 1.0% chitin and at a temperature of 50°C (after the exposure to 80 and 90°C for different times).

ChitMp II_2 lost 60% of its initial activity after 20 min of heating at 80°C, and was completely inactivated after 60 min of heating (Fig. 3a). At 90°C, the enzyme lost 100% of its initial activity after 2 min of heating (Fig. 3).

ChitMp III_2 lost 60% of its initial activity after 20 min of heating at 80°C, and was completely inactivated after 40 min of heating (Fig. 3a). At 90°C, the enzyme lost 100% of its initial activity after 2 min of heating (Fig. 3b).

ChitMp IV_2 lost 90% of its initial activity after 20 min of heating at 80°C, and was completely inactivated after 40 min of heating (Fig. 3a). At 90°C, the enzyme lost 95% of its initial activity after 10 min of heating (Fig. 3b).

Determination of the Ph and Temperature Optima

Experimental design

The STATISTICA 6.0 software was used to generate design matrix and analyze the results. Table II shows the design matrix and responses (results) obtained for the chitinase activity.

TABLE II
Doehlert matrix used for the optimization of chitinase production from *Moniliophthora perniciosa* and results.

Assay	pH	Temperature (°C)	Results ChitMp I (µmol /mL/ min)	Results ChitMp II (µmol glucose/ mL/min)	Results ChitMp III (µmol glucose/ mL/min)	Results ChitMp IV (µmol glucose/ mL/min)
1	5.6	38.7	0.518962	4. 77917	38.88328	88.79409
2	5.6	81.3	0.777343	3.237144	13.13624	89.47147
3	8.4	38.7	2.211738	4.35193	5.787145	55.07999
4	8.4	81.3	1.069247	6.508874	49.93212	167.2721
5	5.0	60	0.042235	0.081851	12.17892	36.07083
6	9.0	60	0.83333	9.049709	28.3441	73.80603
7	7.0	30	0.540504	0.540504	45.96605	41.64516
8	7.0	90	0.475815	2.66952	33.84472	39.07674
9	7.0	60	3.57	9.049	77.5216	174.2154
10	7.0	60	3.57	9.0899	77.45705	172.6066
11	7.0	60	3.599	9.8606	77.80255	176.1628

The results of ChitMp I_2 show that this enzyme is stable between pH 7.0 and 8.3 (Fig. 4a).

In the case of ChitMp II_2, the results show that the enzyme is stable in the range of pH 7.0 - 8.4 and that the activity is higher between 45 and 73°C (Fig. 4b).

In the case of ChitMp III_2, the results show that the optimum activity is between 54 and 67°C and a pH between 7.3 and 7.8 (Fig. 4c).

ChitMp IV_2 showed optimum activity at 58 to 70°C and a pH between 7.6 and 8.2 (Fig. 4d).

Primary Structure Determination (Sequence)

Figure 5 and Table III shows the reads removed from the database of the Genome Project *M. perniciosa* aligned with Seqman / Lasergene (Burland 2000), focusing on the region that includes the catalytic region of the chitinase. The fact that the same sequence is repeated in the three reads (nucleotides 310 to 343, Figure 5b, c and d) indicates that the data submitted are reliable, and all reads exhibit resolved chromatographic peaks. The low peak resolutions can interfere with the identification of nucleotides, with some nucleotides replaced by N, as in position 329 of the read CP02-S3-033-414-B07-UC.F (Fig. 5d). In the same position, reads band c show the nucleotide guanine with peaks of satisfactory quality.

This sequence was aligned to protein sequences of some fungi phylogenetically related to *M. perniciosa* (Lopes 2005) with high identity, particularly in the region of the catalytic site (Fig. 6, Table IV). The fragment of the sequence of chitinase has a shaped molecular mass of 21.62 kDa and a pI of 4.9, reflecting its similarity to chitinases from other fungi (Duo-Chuan 2006) and plants (Wang et al. 2002). This isoelectric point is identical to those found in fungi *Verticillium lecanii* (Zimm.) Viégas and *Piromyces communis* Gold JJ, Heath IB & Bauchop (Wang et al. 2002), and is similar to that reported by Haki and Rakshit (2003).

The sequence of the chitinase consisting of 564 bp and 188 amino acids was not complete, but it contained the conserved region described in the literature as the catalytic region of the enzyme

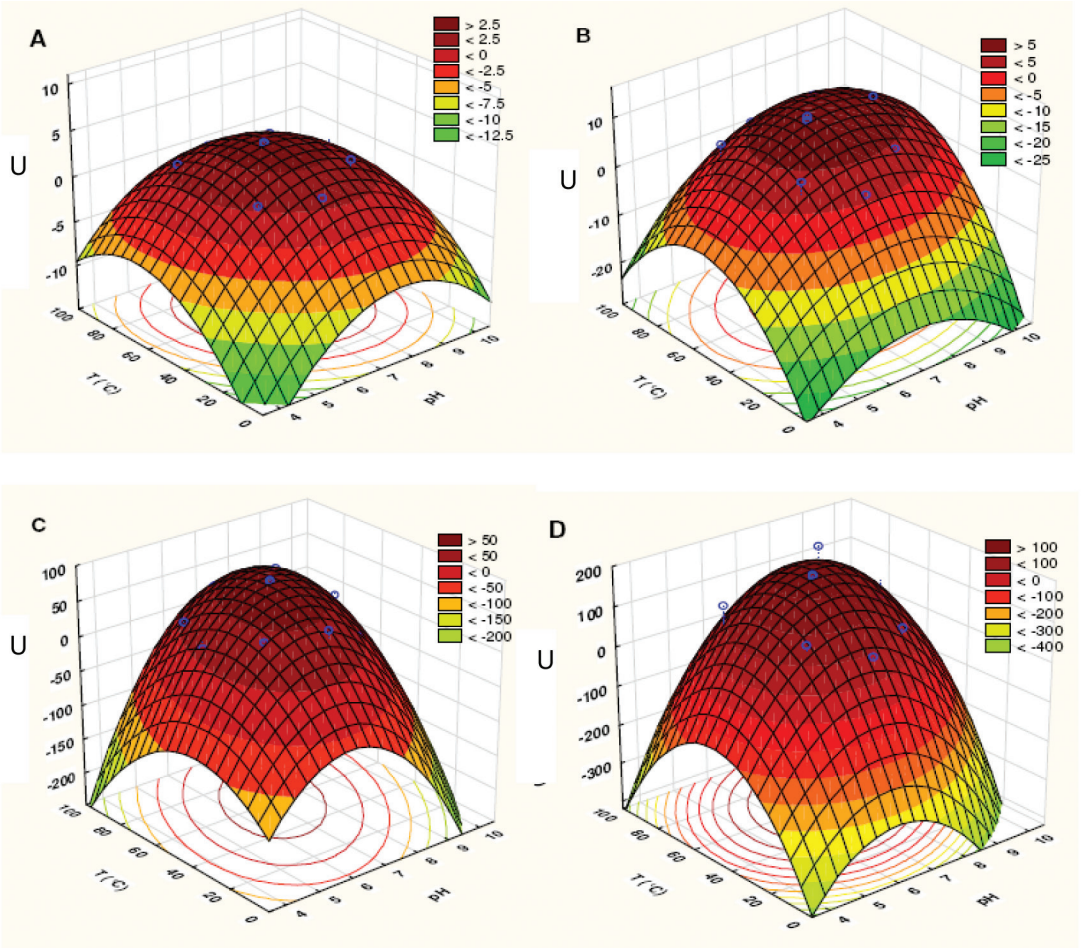

Fig. 4 - Surface plot of the units of activity (U) of *M. perniciosa* as a function of temperature (°C) and pH: **A)** ChitMp I; **B)** ChitMp II; **C)** ChitMp III; **D)** ChitMp IV

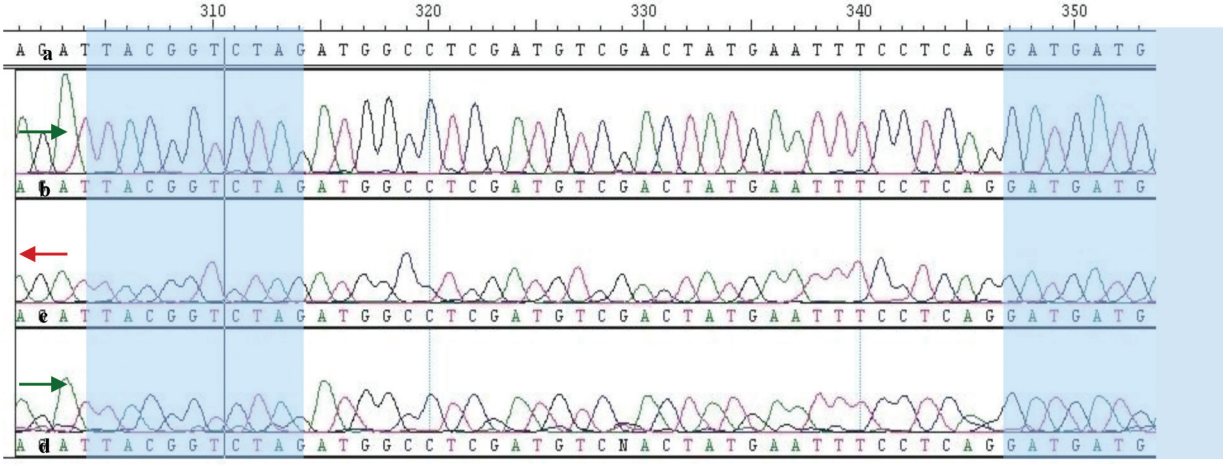

Fig. 5 - Reads removed from the database of the *M. perniciosa* Genome Project aligned with Seqman / Lasergene (Burland 2000), with a focus on the region that includes the catalytic region of the chitinase. In the image, the consensus sequence b corresponds to read CP02-S2-041-309-B06-EM.F; c represents read CP02-S2-039-551-C11-UC.F and read CP02-S3-033-414-B07-UC.F. The green arrows indicate the 5'-3' direction, and the red arrow represents the 3'-5' direction

TABLE III

Reads removed from the database of the *M. perniciosa* Genome Project used for the construction of the contig showing negative energy values and the respective stages of reading.

Reads	E-Value in BLASTx with *Grifola umbellata*	Reads	Nucleotides
CP02-S2-039-551-C11-UC.F	$1e^{-64}$	-3/-2/-1	870
CP02-S2-041-309-B06-EM.F	$2e^{-40}$	+2	853
CP02-S3-033-414-B07-UC.F	$3e^{-52}$	+3	683

Source: Databases were obtained from the Genome Project *M. perniciosa*: <http://www.lge.ibi.unicamp.br/vassoura/>

```
DNA: TCGCCCTTTTCCTTTGCAAGCATTAATCCCGAAACGGGCGAGGTCCAGCTT
 +1: S  P  F  S  F  A  S  I  N  P  E  T  G  E  V  Q  L

DNA: TCTGATAAATGGGCTGATCAAGAAATCCACTACGACGGTGATACATGGGAT
 +1: S  D  K  W  A  D  Q  E  I  H  Y  D  G  D  T  W  D

DNA: GAAGAAGGAAATAACCTATACGGGAATTTGAAGGCTCTCTACAACTTGAAG
 +1: E  E  G  N  N  L  Y  G  N  L  K  A  L  Y  N  L  K

DNA: AAGGAACATCGTCATTTGAAGGTTATGATCTCTATTGGTGGATGGTCATAT
 +1: K  E  H  R  H  L  K  V  M  I  S  I  G  G  W  S  Y

DNA: TCCTCTTCCCTTCACCCTGTCGTTGTCTCTCCTGAGCGCAGGAGAAAGTTT
 +1: S  S  S  L  H  P  V  V  V  S  P  E  R  R  R  K  F

DNA: GTGGAGAGCGCAGTGGCCCTATTGGAAGATTACGGTCTAGATGGCCTCGAT
 +1: V  E  S  A  V  A  L  L  E  D  Y  G  L  D  G  L  D

DNA: GTCGACTATGAATTTCCTCAGGATGATGAACAGGCGTTGGGATATGTACAG
 +1: V  D  Y  E  F  P  Q  D  D  E  Q  A  L  G  Y  V  Q

DNA: CTACTCAAAGAGCTCAGAGAGGCTTTGGACGAGCATGCGAGAACCAAGGAA
 +1: L  L  K  E  L  R  E  A  L  D  E  H  A  R  T  K  E

DNA: ATCGACTATCGCTTTTTGCTCACGGTTGGTTTCCCTTCCCAAAGGTTAGTA
 +1: I  D  Y  R  F  L  L  T  V  G  F  P  S  Q  R  L  V

DNA: TTTGGTTCGTCTCAATATCTTTACAGATCGCTGCACCGTGCGGTTCAGACA
 +1: F  G  S  S  Q  Y  L  Y  R  S  L  H  R  A  V  Q  T

DNA: ATTATAATCGACTTCGTATCAGCGAGATGGATCAGTATCTCGATTTTTGGA
 +1: I  I  I  D  F  V  S  A  R  W  I  S  I  S  I  F  G

DNA: ACA
 +1: T
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Fig. 6 - Conversion of the nucleotide sequence of chitinase from *M. perniciosa* to the amino acid sequence (aa) using the Six Frame Sequence Translator <http://searchlauncher.bcm.tmc.edu/cgi-bin/seq-util/sixframe.pl>. The amino acids that comprise the catalytic site of the enzyme are highlighted.

TABLE IV
Identification of the molds used to construct the model of PDB chitinase *M. perniciosa.*

Method construction	Mold	Identities (%)	Valor-E	Structure
Homology	**2A3E** (RAO et al. 2005)	50	7^{e-42}	chitinase
Homology	**1WNO** (HU et al. 2005)	49	2^{e-41}	chitinase
Threading	**1W9P** (RAO et al. 2005)	50	1^{e-41}	chitinase
Threading	**1ITX** (MATSUMOTO et al. 1999)	33	5^{e-19}	Glycosyl Hydrolase
Threading	**1KFW** (AYATI et al. 2002)	27	5^{e-11}	Chitinase
Threading	**1LL7** (BORTONE et al. 2002)	47	2^{e-37}	Chitinase

(Source: experimental results)

(Synstad et al. 2004, Cederkvist et al. 2007). This region comprises the nucleotides 290 to 323 (Fig. 7).

Localization of the Active Site in the Constructed Models

Construction of three-dimensional model

The results obtained by the construction of the three-dimensional model generated by VMD 1.8.6 (Humphrey et al. 1996) show that the molecular model of chitinase has 188 residues, including 1543 atoms united by 1571 chemical bonds, with 13 hydrogen bonds at the catalytic site. Using the standard cutoff of 3.2 Å, 22 salt bridges were found (Asp31 - Lys45, Asp99 - Lys51 and Glu130 - Arg133). Of the amino acids, 68 are hydrophobic, 47 are polar, 19 are basic and 29 are acidic. The initial model of chitinase had already been satisfactorily validated, but even so, some of the simulations and refinements introduced by molecular dynamics resulted in an updated model as shown in Table V. The model was analyzed by comparative Ramachandran charts, by PROCHECK 3.0 and shows to be improved.

Models of chitinase, built by homology and threading, have been undermined in Amber 8.0 (Case et al. 2004) with 10 sodium ions through the tool. Later, the models were subjected to refinement by 300 and 600 cycles and then were carried out molecular dynamics (MD) by 300, 1000 and 3000 ps in vacuum. For solvent, the models were subjected to refinement by 800 cycles and then were carried out molecular dynamics (MD) by 1000 and 2000 ps.

The validation results show no significant differences between the models of chitinase

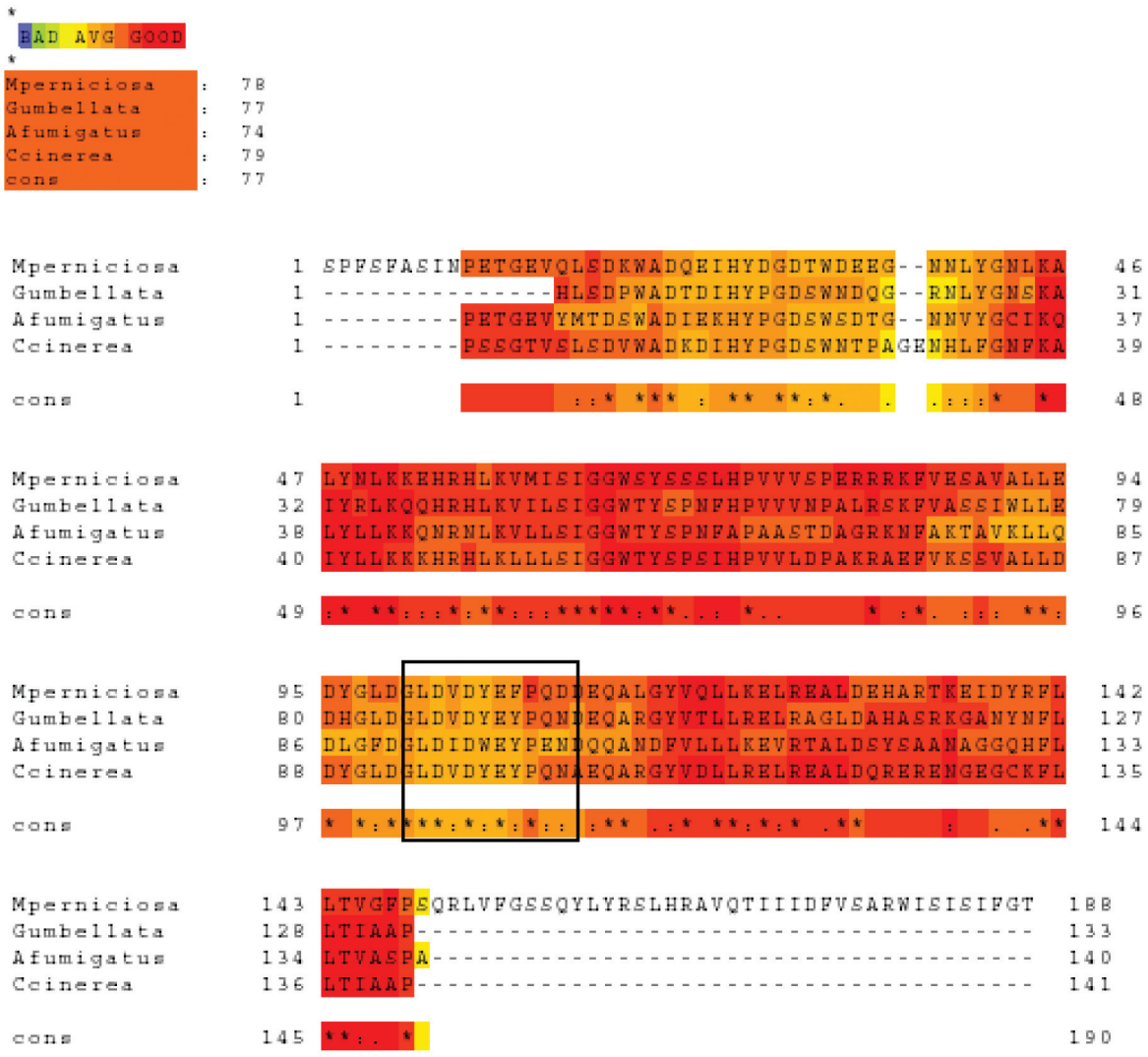

Fig. 7 - Alignment of the sequences of the chitinase gene products of *M. perniciosa*, *Grifola umbelata* (AAO42981.1), *Coprinopsis cinerea* (EAU80760.1) and *Aspergillus fumigatus* (AAO61686.1) through the program TCoffee 5.56, available at <http://www.tcoffee.org/> (Notredame et al. 2000). The region of the catalytic site is noted by a black square.

generated by threading and homology. The model obtained by homology, subject to refinement and 600 cycles called hqmp2, is what generated the best results in the PROCHECK validation (Table V). However, the model suggested for the chitinase of *M. perniciosa* was the model generated by homology with 1000 ps at 300 K in implicit solvent (Table V), which shows the Ramachandran plot, the amino acids Trp66, Ser67, Asp138, Arg151 and Ser157 are in unfavorable regions of the chart and the amino acid Lys51, Ser70, Val75, Asp138 and Asp174 in a region largely favorable, with a score of 94.7% of amino acid residues in favorable local chart Ramachandran (Fig. 8).

The effect of the quality improvement model dm1000_quit can be seen by looking at the side chains, where all parameters analyzed by PROCHECK had lower standard deviations in the chosen model when compared with side chains in the hqmp2 model (Fig. 8). The surface analysis of atomic force (Atomic Mean Force Potential - AMPF) conducted by ANOLEA (data

TABLE V
Summary of the models of the region of the catalytic site of chitinase from *M. perniciosa*, highlighting the results of the Ramachandran validation.

Model	Methodology	Residue Positions (%)	
		Favorable	Unfavorable
hqmp0	Model generated by homology in vacuum Neutralized	98.2	1.8
hqmp1	Model generated by homology Optimized for 300 cycles at 300 K	98.5	1.5
hqmp2	**Model generated by homology in vacuum Molecular Dynamics for 300 ps at 300 K**	**99.4**	**0.7**
hqmp3	Model generated by homology in vacuum Molecular Dynamics for 300 ps at 300 K	95.8	4.2
hqmp4	Model generated by homology in vacuum Molecular Dynamics for 1000 ps at 300 K	89.3	10.7
hqmp5	Model generated by homology in vacuum Molecular Dynamics for 3000 ps at 300 K	87.5	13.5
tqmp0	Model generated by threading in vacuum Neutralized	97.6	2.4
tqmp1	Model generated by threading in vacuum Optimized for 300 cycles at 300 K	98.1	1.9
tqmp2	Model generated by threading in vacuum Optimized for 600 cycles at 300 K	98.0	2.0
tqmp3	Model generated by threading in vacuum Molecular Dynamics for 300 ps at 300 K	95.8	4.2
tqmp4	Model generated by threading in vacuum Molecular Dynamics for 1000 ps at 300 K	91.1	8.9
tqmp5	Model generated by threading in vacuum Molecular Dynamics for 3000 ps at 300 K	65.8	34.2
hq_neut	Model generated by homology in solvent Optimized for 800 cycles at 300 K	98.8	1.2
dm1000_ quit	**Model generated by homology in solvent Molecular Dynamics for 1000 ps at 300 K**	**94.7**	**5.3**
dm2000	Model generated by homology in solvent Molecular Dynamics for 2000 ps at 300 K	90.5	9.5

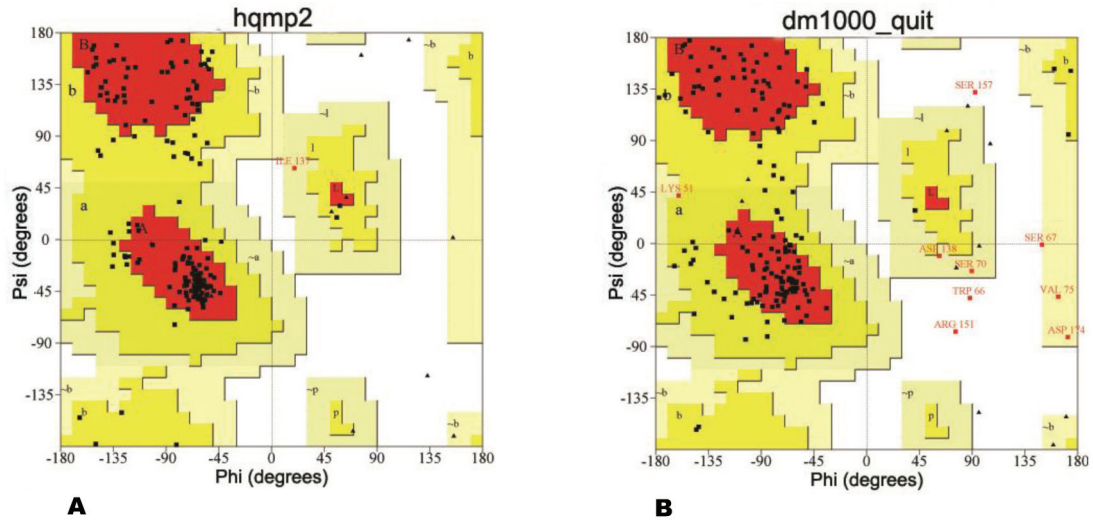

Fig. 8 - Ramachandran plots of the chitinase models showing the amino acid residues in favorable positions as red and yellow regions and those in unfavorable positions as white regions. A) Model before optimization by 600 cycles; and B) the final model simulated by 1 ns of molecular dynamics.

not shown) also validated the models; however, the chosen model, dm 1000_quit, had a better AMPF and a more negative energy than other models constructed by comparative modeling, supporting the validation performed by PROCHECK. The models generated by MD 300, 1000 and 3000 ps did not show satisfactory results when validated by ANOLEA, demonstrating a positive energy (data not shown), and models generated by MD 300, 1000 and 3000 ps using the threading methodology were also not satisfactory. For these reasons, the dm1000_quit was chosen as the best model for the chitinase of *M. perniciosa* (Fig. 9).

The motif LDVDYEFP, corresponding to Leu98, Asp99, Gly100, Leu101, Asp102, Val103, Asp104, Tyr105, Glu106, Phe107 and Pro108 and described as the catalytic site, corresponds to residues 98-108 (Fig. 10) that participate in the formation of a β-sheet, a structural form that is easily recognized as part of a more complex system. A conserved region designed SIGG (Fig 6), corresponding to Ser62, Ile63, Gly64 and Gly65, also were found in this model it is a important represents hydrophobic domain to catalysis (Zhu et al. 2008). The final structure of the model has seven α-helices, seven β-sheets and fourteen turns. Antiparallel β-sheets can be composed of several strands that can be distributed in various parts of the sequence, and can be composed of hydrophilic (Tyr107, Asp99, Asp102, Asp104 and Glu106) or hydrophobic amino acids (Val103, Trp105 and Pro108) (Höltje et al. 2003).

Characteristics of Model Generated by Homology in Solvent 1000 ps:

The molecule possesses 1543 atoms united by 1571 chemical bonds and 13 hydrogen bonds at the catalytic site. In VMD 1.8.6 analysis with a standard cutoff of 3.2 Å, 22 salt bridges were found. Salt bridges and hydrogen bonds are important elements contributing to the molecular stability and substrate specificity, respectively. The overall molecular structure consists of six α-helices, two β-sheets and eight turns.

Ramachandran plot analysis showed that 91.7% of amino acid residues were found in favorable areas in the model generated in solvent after 1 ns

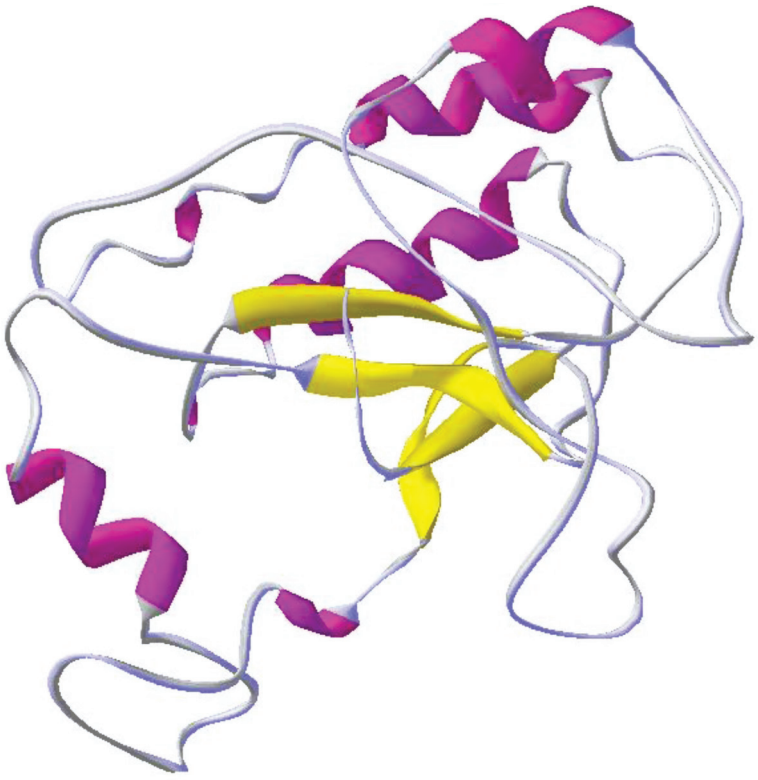

Fig. 9 - Three-dimensional model of *M. perniciosa* chitinase

of dynamics, resulting in an RMS of 2.68 Å. The molecular Dynamics for 2 and 3 ns (data not shown), showed less satisfactory results than the 1 ns case. The Ramachandran plot of the initial model presents the best values as a result of the good template (50% identity).

DISCUSSION

Chitinase is one of the most important cell wall lytic enzymes; it has been purified from a number of fungal species and found to have differing physiological properties, molecular structures, and molecular weights.

In the studies made by Wang et al. (2002), the enzymes lost >50% of their initial activities after only 3 min of heating at 100°C, and were completely inactivated after 9 min of heating.

The chitinase from *Isaria japonica* lost 0% of its activity at 50oC (Kawachi et al. 2001). The chitinase from *Aspergillus fumigatus* lost 30% of its activity after 60 minutes of heating at 55°C (Xia et al. 2001). The chitinase from *Trichoderma harzianum* was inactivated at 60°C (De La Cruz et al. 1992).

In this study, the temperature and pH optima of ChitMp I and II were similar, indicating that these enzymes act synergistically. However, ChitMp III and IV showed similar characteristics that were different from those of ChitMp I and II.

Miyashita et al. (1991) found three chitinases, ChiA, ChiB, ChiC and ChiD in *S. lividans*. ChiC and ChiD exhibited similar activities and generic structures, but ChiA and ChiB differ.

Felse and Panda (1999a, b) reveal that the three chitinases of *T. harzianum* (CHIT33, CHIT37 and CHIT42) did not result from the proteolytic breakdown of a single common chitinase, but rather were transformed from three different genes with little or no homology.

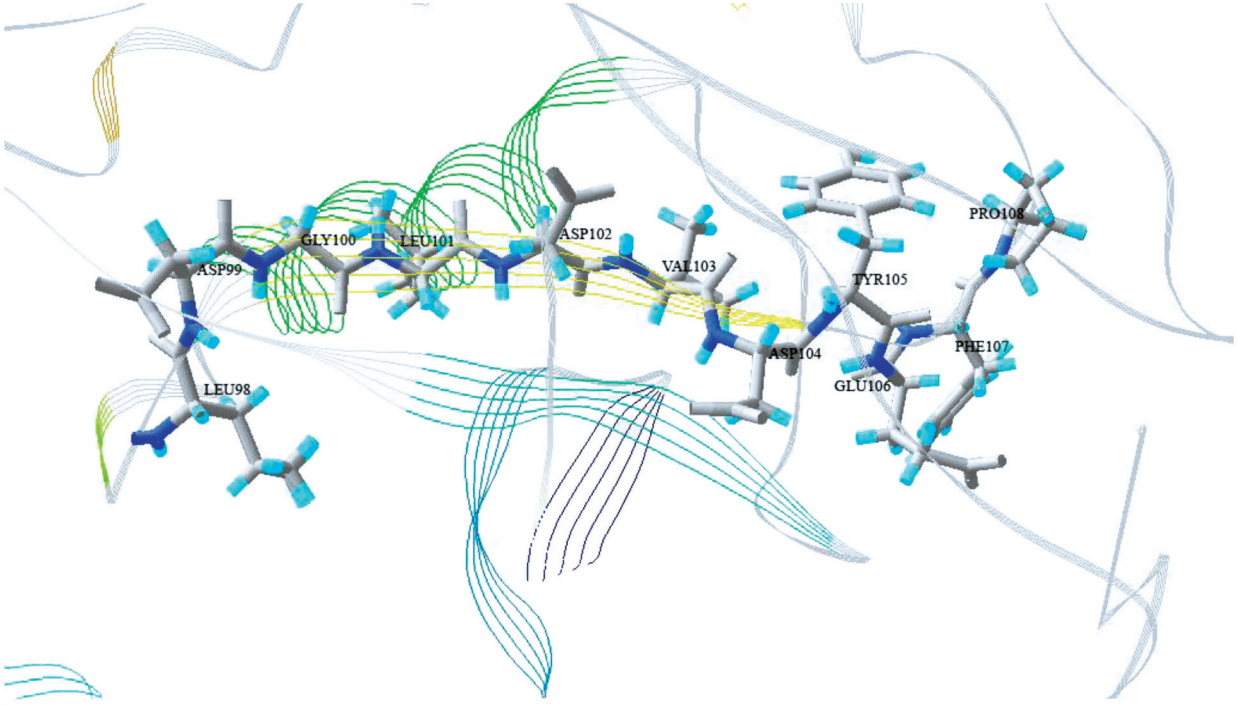

Fig. 10 - The LDGLDVDYEFP of Leu98, Asp99, Gly100, Leu101, Asp102, Val103, Asp104, Tyr105, Glu106, Phe107 and Pro108 described as the catalytic site of the 3D model of *M. perniciosa* chitinase.

Duo-Chuan et al. (2005) found similar characteristics in relation to temperature (between 30 and 40°C) and pH (4.0 - 8.0) in the two chitinases from *Talaromyces flavus* (Klöcker) Stolk & Samson.

The optimal temperature for many fungi is in the range between 20 and 40°C, except for some thermophilic fungi that have maximal activities around 55 and 70°C, such as *Thermomyces lanuginosus* Tsikl. (Guo et al. 2005) and *Talaromyces emersonii* Stolk (Mccormack et al. 1991). The chitinase from *A. fumigatus* YJ-407 retained 40% of its activity after heating at 60°C for 30 min, and these results indicated that this enzyme was the most thermostable among the fungal chitinases reported (Xia et al. 2001).

In summary, the chitinase from *M. perniciosa* exists in several isoforms. One of these isoenzymes (ChitMp IV) showed high thermostability.

Compared with the modeling results, data obtained in silico from the database of the *Moniliophthora perniciosa* Genome Project provided the region of the nucleotide sequence of the chitinase around the catalytic site of the enzyme. This region comprises 564 bp and 188 amino acids. These results were sufficient to generate a 3D model of the enzyme with 50% similarity with a model obtained from the program Deep View v3.7.

The modeled fragment includes the conserved domains SXGG (Wattanalai et al. 2004) and DxDxE (Watanabe et al. 1992, Synstad et al. 2004, Cederkvist et al. 2007), where the latter region is only found in Family 18 chitinases (Okazaki et al. 2004, Cederkvist et al. 2007). In *Nomuraea rileyi* (Farl.) Samson, this sequence corresponds to EDGIDIDWE (Wattanalai et al. 2004), in *Rhizopus oligosporus* Saito to DGFDFDIE (Takaya et al. 1998), in *Trichoderma harzianum* to LDGFDLDNE (Hoell et al. 2005) and in *Moniliophthora perniciosa* to LDGLDVDYEFP,

and other conserved domains have been identified, such as SIGG (residues 62 to 65). In all these organisms, the LXXLDXDXE motif is cited as the catalytic site of the enzyme (Lu et al. 2002).

Studies by Lu et al. (2002) on *Manduca sexta* chitinase reveal the essential nature of residues Glu146 to Glu106 which are equivalent to Asp144 and 142 to Asp104, as well Asp 104 and 102 in the chitinase of *M. perniciosa* for the catalytic activity of the enzyme. Their results suggest that Glu146 is more critical than Asp142 and Asp144 to enable the participation in the first stage of the mechanism of catalysis. According to Synstad et al. (2004), a mutation in Asp142 is highly deleterious for the catalytic activity of the enzyme, and according to Lu et al. (2002), a mutation in Asp144 results in a 70% loss of enzyme activity.

From the 3D model of the chitinase and the nucleotide sequence determined by PCR, sequencing and cloning, one of the goals of future works will be to select a specific inhibitor by docking with this enzyme.

ACKNOWLEDGMENTS

This work was supported by Financiadora de Estudos e Projetos (FINEP), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB). We also thank the Programa de Pós Graduação em Biotecnologia (PPGBiotec-UEFS).

RESUMO

A enzima quitinase de *Moniliophthora perniciosa*, o fungo causador da doença vassoura de bruxa em *Theobroma cacao*, foi parcialmente purificada por precipitação com sulfato de amônio e filtração em coluna de Sephacryl S-200 usando fosfato de sódio como tampão de extração. A metodologia de Superfície de resposta (MSR) foi usada para determinar o pH e temperatura ótimos. Foram isoladas quatro quitinases diferentes: ChitMp I, ChitMp II, ChitMp III e ChitMp IV. A ChitMp I mostrou temperatura ótima de 44-73°C e pH ótimo de 7,0-8,4. A ChitMp II apresentou temperatura ótima entre 45-73°C e pH ótimo de 7,0-8,4. ChitMp III apresentou temperatura ótima de 54-67°C e pH ótimo de 7,3-8,8. ChitMp IV apresentou temperatura ótima de 60°C e pH ótimo em 7,0. Em relação à biologia computacional a sequência primária foi feita *in silico* usando banco de dados do Projeto Genoma/Proteoma do *M. perniciosa*, obtendo-se uma sequência com 564 pb e 188 aminoácidos, que foi utilizada para a obtenção da estrutura tridimensional por metodologia de modelagem comparativa. Os modelos gerados foram submetidos à validação usando Procheck 3.0 and ANOLEA. O modelo proposto para a quitinase foi submetido à análise dinâmica por 1 ns, resultando em um modelo com 91,7% de seus resíduos em locais regiões favoráveis no gráfico de Ramachandran e RMS de 2,68.

Palavras-chave: quitinase, *Moniliophthora perniciosa*, caracterização cinética, purificação, isoenzimas, estabilidade térmica, estrutura 3D, modelagem comparativa.

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