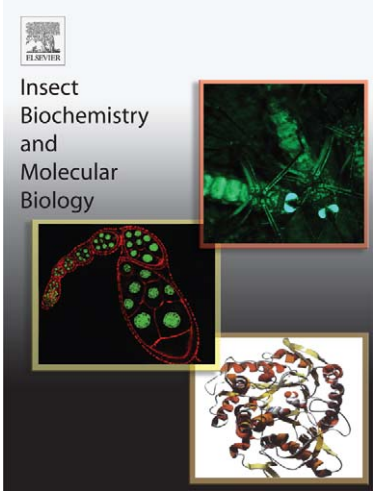

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Purification, characterization and molecular cloning of the major chitinase from *Tenebrio molitor* larval midgut.

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ABSTRACT

Insect chitinases are involved in degradation of chitin from the exoskeleton cuticle or from midgut peritrophic membrane during molts. cDNAs coding for insect cuticular and gut chitinases were cloned, but only chitinases from moulting fluid were purified and characterized. In this study the major digestive chitinase from *T. molitor* midgut (TmChi) was purified to homogeneity, characterized and sequenced after cDNA cloning. TmChi is secreted by midgut epithelial cells, has a molecular weight of 44 kDa and is unstable in the presence of midgut proteinases. TmChi shows strong substrate inhibition when acting on umbelliferyl-derivatives of chitobio- and chitotriosaccharides, but has normal Michaelis kinetics with the N-acetylglucosamine derivative as substrate. TmChi has very low activity against colloidal chitin, but effectively converts oligosaccharides to shorter fragments. The best substrate for TmChi is chitopentaose, with highest k_{cat}/K_M value. Sequence analysis and chemical modification experiments showed that the TmChi active site contains carboxylic groups and a tryptophane, which are known to be important for catalysis in family 18 chitinases. Modification with p-hidroximercuribenzoate of a cysteine residue, which is exposed after substrate binding, leads to complete inactivation of the enzyme. TmChi mRNA encodes a signal peptide plus a protein with 37 kDa and high similarity with other insect chitinases from family 18. Surprisingly, this gene does not encode the C-terminal Ser-Thr-rich connector and chitin-binding domain normally present in chitinases. The special features of TmChi probably result from its adaptation to digest chitin-rich food without damaging the peritrophic membrane.

Key words: midgut chitinase, coleopteran chitinase, chitin binding domain, chitinase characterization, chitinase sequencing, chitin digestion, peritrophic membrane.

1. INTRODUCTION

Chitinolytic enzymes are enzymes that act on chitin, the β-1,4-homopolymer of N-acetylglucosamine. Chitinolytic enzymes include (Kramer and Koga, 1986): chitinase (E.C. 3.2.1.14), which catalyses the random hydrolysis of internal bonds in chitin forming smaller oligosaccharides and β-N-acetyl-D-glucosaminidase (E.C. 3.2.1.52), which liberates N-acetylglucosamine from the non-reducing end of oligosaccharides. Chitinolytic enzymes associated with the ecdysial cycle have been demonstrated to act synergiscally in cuticular chitin degradation (Kramer and Koga, 1986).

In the last years several insect chitinases belonging to family 18 of glycoside hydrolases (Coutinho and Henrissat, 1999) have been cloned and sequenced, but few of these enzymes were studied in detail (Terra and Ferreira, 2005). The best-known insect chitinase is the molting fluid chitinase from the lepidopteran *Manduca sexta*. The enzyme has a multidomain architecture that includes a signal peptide, an N-terminal catalytic domain, with the conserved sequence (F/L)DG(F/L/I)D(L/I)DWEYP, and a C-terminal cysteine-rich chitin-binding domain (CBD), which are connected by the interdomain serine/threonine-rich O-glycosylated linker (Arakane *et al.*, 2003). The residues D142, D144, W145, and E146 of the consensus sequence have been shown by site-directed mutagenesis to be involved in catalysis (Lu *et al.*, 2002; Zhang *et al.*, 2002).

Several insect chitinases have been described as gut chitinases, because they were cloned and sequenced from cDNA libraries prepared from midgut cells and expression of the corresponding genes were localized in the midgut (Shen and Jacobs-Lorena, 1997; Girard and Jouanin, 1999; Ramalho-Ortigão and Traub-Cseko, 2003; Bolognesi *et al.*, 2005). The proposed roles of those chitinases are: to act in concert with a chitin synthase to modulate the thickness and permeability of the peritrophic membrane in *Anopheles gambiae* (Shen and Jacobs-Lorena, 1997) and *Lutzomya longipalpis* adults (Villalon *et al.*, 2003) or to carry out the intermolt digestion of the peritrophic membrane in *Spodoptera frugiperda* larvae (Bolognesi *et al.*, 2005). The role of chitinase in *Phaedon cochleariae* is assumed to be similar to that in *A. gambiae* (Girard et Jouanin, 1999), in spite of the lack of direct evidence.

Extensive damage of the peritrophic membrane causes significant reduction in insect growth due to impaired nutrient utilization (Terra and Ferreira, 2005). Because of this, the addition of chitinase to insect diets decreases insect performance (Otsu *et al*., 2003; Fitches *et al.*, 2004). The same result is observed in insects feeding on transgenic plants expressing chitinase (Ding *et al.*, 1998).

The early observation that there is a positive correlation between midgut chitinase activity and a chitin-rich diet in several insects (Terra and Ferreira, 1994) is unexpected on the ground of research reviewed above, regarding chitinase damages to peritrophic membrane. In the same direction, the recent finding that *Aedes aegypti* larvae digest dietary chitin is also unexpected (Souza-Neto *et al.*, 2003). The lack of detailed data on insect gut chitinase precludes shedding light on the problem.

This paper describes the purification and characterization of the *T. molitor* larval midgut chitinase and the cloning of its corresponding cDNA. Some characteristics of this enzyme, like its oligochitosaccharidase activity and the lack of a CBD, are probably related to the adaptation of this chitinase to digest chitin structures in food without damaging the peritrophic membrane.

2. MATERIALS AND METHODS

2.1. Animals and chemicals.

Stock cultures of *T. molitor* were maintained under natural photoregime conditions on wheat bran at 24–26°C and 70–75% relative humidity. Fully-grown larvae of both sexes (each weighing about 0.12 g), having midguts full of food, were used.

Chitooligosaccharides - chitobiose (C2), chitotriose (C3), chitotetraose (C4), chitopentaose (C5), and chitohexaose (C6) - were purchased from Calbiochem (USA) or Seikagaku (Japan), whereas the other substrates were acquired from Sigma (USA). All chemical substances used were of analytical grade.

2.2. Preparation of samples.

Larvae were immobilized by placing them on ice, after which they were dissected in cold 342 mM NaCl. For determination of enzymes in gut portions, tissues were homogenized in cold MilliQ water with the aid of a Potter–Elvehjem homogenizer with 10 strokes and centrifuged at 10,000 g for 10 min at 4°C. The pellets were homogenized in cold MilliQ water using a micro tube homogenizer (Model Z 35, 997-1, Sigma, USA). The homogenates were stored at -20ºC up till at leasta month without noticeable changes in the activities assayed. Both pellets and supernatants were assayed.

For chitinase purification, the midgut was pulled apart and the anterior two thirds of the midgut with contents were removed and homogenized as before in cold 20 mM Tris-HCl buffer

pH 7 containing 1 mM transepoxysuccinyl-L-leucyl-amido (4-guanidino butane) (E64), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM phenylthiocarbamide (PTC). The homogenate was centrifuged as before, the supernatant was filtered through a PVDF membrane with a pore size of 0.44 μm (Millipore) and used immediately.

Wheat bran was homogenized in cold MilliQ water using a homogenizer model Skymsem TAR-02 (Siemsen, Brazil) at 10,000 rpm for 3 cycles of 30 s. The homogenate was sonicated with a Branson Sonifier 250, using three cycles of 30 s each (output 3) with 30 s intervals. The sample, after homogenization as described before, was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was passed through glass wool to be freed of fat.

2.3. Protein determination and hydrolase assays.

The samples were dialyzed for 3 h at room temperature against 20,000 volumes of 10 mM Tris, containing 10 mM Na_2CO_3, 0.75% Tween 20, with pH 10-12, before protein determination with the silver method of Krystal *et al.* (1985), using ovalbumin as a standard.

Chitinase activity was determined by measuring the release of reducing groups (Noelting and Bernfeld, 1948) from 0.5% (w/v) colloidal chitin (CC), or the release of 4-methylumbelliferone (Baker and Woo, 1992) from 0.1 mM methylumbelliferyl-β-N',N',N'-triacetyl-chitotrioside (MUC3), 0.1 mM methylumbelliferyl- β -N',N'-diacetyl-chitobioside (MUC2) and 0.1 mM methylumbelliferyl- β -N'-acetylglucosamine (MUNAG). Colloidal chitin was prepared according to Hsu and Lockwood (1975).

N-acetyl-glucosaminidase activity was determined by measuring the release of *p*-nitrophenolate (Terra *et al.*, 1979) from 10 mM *p*-nitrophenyl- β -D-N'-acetyl-glucosaminide (p-NAG).

Unless otherwise specified, all substrates were assayed in 50 mM sodium citrate pH 5.0 at 30 °C, under conditions that activity was proportional to protein concentration and to time. Controls without enzyme or without substrate were included. One unit of enzyme (U) is defined as the amount that hydrolyses 1 μmol of bonds/min.

2.4. *T. molitor* chitinase (TmChi) purification

To 1 mL of the midgut supernatant, solid $(NH_4)_2SO_4$ was added to a final concentration of 0.5 M (6.6% w/v or 12.5% saturated). This sample was then applied onto a 1 mL HiTrap Phenyl hydrophobic interaction chromatography (HIC) column (FPLC System, Pharmacia, Sweden)

equilibrated with 20 mM Tris-HCl buffer pH 7.0, containing 0.5 M $(NH_4)_2SO_4$, 1 mM PMSF, and 1μM E64. After passing 5 mL of the last buffer through the column, elution was accomplished with 0.3 – 0 M $(NH_4)_2SO_4$ gradient in the same buffer (20 mL) plus 5 mL of buffer without $(NH_4)_2SO_4$. The flow was 1 mL/min and fractions of 1 mL were collected. The more active fractions against MUC3 were pooled (28-29), $(NH_4)_2SO_4$ was added to attain a concentration of 0.5 M and the material was loaded onto a 1 mL Resource Phenyl column (FPLC System, Pharmacia, Sweden) equilibrated and eluted as before. Fractions of 0.4 mL were collected. Fractions more active against MUC3 (69–71) were combined and applied onto a 1 mL Resource Q column (Pharmacia, Sweden) equilibrated in 20 mM Tris-HCl buffer pH 7.0, containing 1 mM PMSF and 1 μM E64. After passing 5 mL of this buffer through the column, elution was accomplished with 0.3 - 0.35 M NaCl gradient (20 mL). The flow was 1 mL/min and fractions of 0.4 mL were collected. Fractions more active against MUC3 (23-26) were pooled, diluted 5 times with the same buffer and reapplied onto the same column and eluted as described above. Fractions more active (25-27) were combined and used as purified chitinase (TmChi). Screening of activity against MUC3 in fractions obtained during purification was done in 20 mM Tris-HCl buffer pH 7.0

2.5. Determination of molecular masses by gel filtration.

TmChi was applied onto a HR 10/10 Superdex 75 column (Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl pH 7.0. Proteins were eluted with the same buffer (30 mL), with a flow of 1 mL/min, and fractions of 0.4 mL were collected. Molecular mass standards used were ribonuclease A (13.7 kDa), soybean trypsin Inhibitor (21.5 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and thyroglobulin (669 kDa).

2.6. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and detection of proteins and enzyme activity in the gel.

SDS-PAGE was accomplished in slab gels according to Laemmli (1970) as detailed in Ferreira et al. (2001). Staining for protein was done with the silver method of Blum *et al*. (1987). Molecular masses were calculated according to Shapiro *et al*. (1967). The following mass standards were used: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97.4 kDa).

Non-denaturating PAGE was used for *in gel* assays of chitinase. The gel contained 7 to 12% acrylamide. The samples were not heated and no β-mercaptoethanol was added. After the run, the gel slab was maintained in 20 mM acetate buffer pH 5.0 for 15 min (buffer changes each 5 min) and then placed in a semi-dry system at 30 °C with a piece of paper wetted with a 0.1 mM MUC3 in the same buffer. After 1-5 min, the paper was removed and the gel was observed in an UV transilluminator. Activity appears as white bands against the dark background and are approximately the same as before electrophoresis. The following molecular mass standards were used: soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa). Molecular masses were calculated using Ferguson plots (see Andrews, 1986).

2.7. Microsequencing of purified chitinase

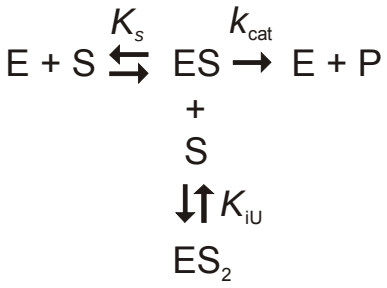
Samples with 40 μg of purified TmChi were concentrated in a vacuum desiccator (HetoLab Equipment, Denmark) and dissolved in 62.5 mM Tris–HCl buffer pH 6.75, containing 2% (w/v) SDS, 5% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol and 0.001% (v/v) bromophenol blue. After a pre-run (10 mA, 30 min), with 0.1 M sodium thioglycolate in the running buffer, the samples and pre-stained standards were loaded and electrophoretically resolved as described above. The single peptide in the gels was electroblotted onto polyvinylidene difluoride (PVDF) membranes according to Matsudaira (1987). The PVDF membranes were stained for proteins using 0.1% Coomassie Blue R-250 in a 50% (v/v) methanol solution, and destained with a 50% methanol solution. Dried PVDF membranes were the source of the peptide for N-terminal microsequencing. Sequence analyses were performed at the laboratory of Prof. Luiz Juliano Neto at INFAR, UNIFESP (São Paulo, Brazil) with a peptide sequencer from Applied Biosystems.

2.8. Kinetic studies

The effect of substrate concentration on TmChi activity was studied by using at least 10 different substrate concentrations. *K*m and *V*max values (mean and SEM) were obtained by the least-squares method using the software Enzfitter (Elsevier, Biosoft). TmChi K_{iC} values were determined after assays with at least ten different concentrations of substrate (MUNAG) in each of at least five different concentrations of substrate tested as inhibitor (chitooligosaccharides, degree of polymerization ranging from 2 to 6). K_{iC} values were calculated from plots of slopes of

Lineweaver–Burk plots against inhibitor concentration (Segel, 1975), using the Enzfitter software.

When inhibition by high substrate concentration was observed, kinetic parameters were calculated taking into account the equilibria and equation below (Segel, 1975):



$$v = \frac{V_{max}[S]}{K_s + [S] + \frac{[S]^2}{K_{iU}}}$$

The effect of pH on enzyme stability and on enzyme activity was evaluated with the following buffers (25 mM): sodium acetate (pH 3.5–5.5), sodium phosphate (pH 6–8), Tricine (pH 8–9). The pH of the buffers was adjusted at the assay temperature (30 °C) and to a final ionic strength equivalent to 0.1 M NaCl. In order to determine enzyme stability in different pHs, enzyme samples were previously maintained at 30^0C for 120 min at several different pHs, before moving to pH 7 for standard assay. The activities were compared to a control assayed at pH 7 that was taken as 100. The p*K*s of catalytical groups in the free enzyme (pK_E) and in the enzyme–substrate complex (pK_{ES}) were calculated (Segel, 1975) from K_M and V_{max} values determined at 19 different pHs with seven MUNAG concentrations.

2.9. Capillary Electrophoresis of TmChi Products

A sample containing purified TmChi (0.37 ηM) was added to 10 μM of each chitooligosaccharide at 30°C in 100 μM citrate buffer pH 5. After stopping the reaction and concentrating the sample 100 times, TmChi products were detected by capillary electrophoresis. At low substrate concentrations, k_{cat}/K_M values can be directly calculated from the velocities of product release.

The capillary electrophoresis was performed in a equipment made at Analytical Instrumentation Laboratory at the Chemistry Department of São Paulo University (da Silva *et al.*, 2002). The temperature inside the apparatus was 30^oC. Fused-silica capillary (50μm ID and 360μm OD) (from J&W Scientific, Folsom, CA, USA) was used to carry the experiments. The total length of the capillary was 72.5 cm and the effective length 62.5 cm. Product detection was

performed with a contactless conductivity detector at 600 kHz and 2 Vpp voltage signal (da Silva and do Lago, 1998).

Before the runs, the capillaries were connected to a vacuum pump and washed by this sequence of 20 min flushes: 0.1 M NaOH, water, and running electrolyte [10 mM NaOH, 200 μM cetyltrymethylammonium bromide (CTAB), 4.5 mM Na_2HPO_4, 10% (v/v) acetonitrile]. Before the first injection, high voltage was applied for approximately 20 min. Between the runs, the capillary was flushed with the electrolyte for about 2 min. The potential applied to separation was 15 kV, positive pressure (14.7 mmHg , 30s) was used to inject the samples and 1 mM methionine was used as electrophoretical standard. Calibration curves were prepared with each of the chitooligosaccharides studied.

2.10. Chemical modification studies.

TmChi chemical inactivation was attempted with 4-(hydroxymercuri)benzoic acid (pHMB), diethyl pyrocarbonate (DPC), tetranitromethane (TNM), N-bromosuccinimide (NBS), 1-phenylglyoxal (PG), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) or ethylenediaminetetraacetic acid (EDTA). pHMB and EDC react, respectively, with sulphydryl and carboxylate (Carraway and Koshland Jr., 1972) groups. DPC, TNM, NBS and PG react with His (Miles, 1977), Tyr (Riordan and Vallee, 1972), Trp (Spande and Witkop, 1967) and Arg (Takahashi, 1968) lateral chains, respectively. EDTA chelates divalent cations. TmChi remaining activity after different reaction times were measured using 10 mM MUNAG as substrate.

The inactivation reactions were performed with 10 mM of each reactant, except EDC and pHMB, in 100 mM sodium citrate buffer, pH 5.0. In the modification reaction with EDC, the buffer used was 100 mM N,N,N',N'- tetramethylethylenediamine (TEMED) pH 5.0, containing 100 mM glycine ethyl ester and when pHMB was present, the buffer was 50 mM sodium citrate pH 5.0. Prior to chemical modification with EDC, the enzyme was dialyzed in the buffer used for the modification reaction and reactions were stopped by adding a volume of 100 mM sodium citrate, pH 5. Other chemical modifications were stopped by diluting the reaction mixture with a solution of the target amino acid in a concentration 10 times higher than the reactant. In experiments with EDTA, $CaCl_2$ was added. Controls of enzyme activity in the absence of chemical modifiers showed that the enzyme is stable in all the conditions used.

2.11. Cloning and sequencing of the cDNA that codes for TmChi.

Total RNA was extracted from midgut epithelium of *T. molitor* larvae with Trizol following the instructions of the manufacturer, Invitrogen, which are based on Chomczynski and Sacchi (1987), and sent to Stratagene (La Jolla, CA), in order to construct a cDNA library. At Stratagene the mRNAs were isolated, divided into two equal samples and used in cDNA synthesis with a poly-T and a random primer. Finally, the two cDNA pools were mixed (1:1) and non-directionally inserted in the vector l ZAPII. The library titer is $1.5.10^{10}$ pfu ml^{-1}.

Chitinase cDNA was obtained during random sequencing of *T. molitor* library. A 3´ fragment that coded a peptide with high similarity to chitinases in GenBank was obtained and, in order to have the full sequence, primers was designed to cover the 5´ region. A PCR reaction using T3 primers combined with *T. molitor* chitinase specific primer (5´ GCTGGTGCTCCGACTTCGT 3´) was done using TAQ Polimerase (Invitrogen, USA) (5 units) in 20 mM Tris–HCl buffer, pH 8.4, with 50 mM KCl, 0.2 mM dNTP, and 4 mM $MgCl_2$. The amplification was reached using 30 cycles at the following conditions: 1 min at 94 ºC, 1 min at 52 ºC and 1 min at 72 ºC. The product of PCR reaction was cloned in pGEM-T Easy Vector (Promega, USA) and sequenced.

DNA sequencing was performed with the DNA kit Big Dye Terminator Cycle sequencing (PE Applied Biosystems). Each clone was sequenced in both DNA strains. The electropherograms of the sequenced clones were automatically processed for base calling, low quality detection and vector trimming, and assembled using the algorithm Phred-Phrap (http:// www.phrap.org/phredphrapconsed.html) (Ewin et al., 1998; Ewin and Green, 1998). The quality of the complete assembled chitinase was above 50.

2.13. Chitin binding assays

Chitin binding assays were done according to Arakane *et al.*, 2003. Crude chitinase preparations from *Serratia marcescens* (Sigma, C7809) and *Streptomyces griseus* (Sigma, C6137) were used as controls. Experiments were performed 3 times, with 7 μU of TmChi, 29 μU of *S. griseus* chitinase or 44 μU of *S. marcescens* chitinase. Enzymes were incubated with 0.5 mg of colloidal chitin in 50 μL of 10 mM sodium phosphate buffer pH 8.0. The mixture was incubated at room temperature for 1h and then centrifugated for 3 min at 14,000 g. Soluble and bound chitinase activities were measured with MUC3 as substrate.

3. RESULTS

3.1. Chitinase activity in *T. molitor* larval midgut.

Chitinolytic activity is higher in *T. molitor* midgut than in other insects (Table 1), whereas no activity was detected in the Orthoptera *Abracris flavolineata*. Wheat flour, used to feed the larvae, has negligible activities against the synthetic substrates used, but displays significant activity against colloidal chitin (not shown). Nevertheless, *T. molitor* midgut chitinolytic activity is stable at pH 3 (pH optimum 4), whereas wheat flour activity inactivates below pH 4 (data not shown).

Chitinase and N-acetyl-glucosaminidase activities are found in the lumen of *T. molitor* anterior midgut, lesser amounts in posterior midgut and negligible quantities in the hindgut (Table 2). Since only one activity against MUC3 is found after non-denaturating PAGE (41 kDa) (data not shown), this shows that a single molecular species is responsible for chitinase activity in *T. molitor* midgut.

The chitinase activity present in midgut cells indicates that the enzyme may be secreted by the insect. This is also suggested by the adaptative increase in midgut chitinase activity observed when *T. molitor* larvae are reared in the presence of antibiotics or in axenic conditions (Genta *et al*., 2006).

3.2. Purification of *T. molitor* chitinase (TmChi).

The recovery of purified TmChi was increased when phenylthioureia was present in the homogenization media at pH 7.0 (pHs from 5 to 9 were tested). Homogenization of *T. molitor* midgut with proteinase inhibitors (E64 and PMSF) is essential for recovering TmChi. In the absence of those inhibitors in any purification step, TmChi is degradated and several activity peaks corresponding to TmChi fragments still active are seen in hydrophobic and anion-exchange chromatographies. Those fragments have 10-30 kDa and are very unstable at 4ºC. Purified chitinase is unstable to freezing and thawing, but after being passed through a 0.22 μm filter membrane (Millex GV JBR6 10021, Millipore) it is stable at 4ºC for at least 4 weeks.

Stepwise chromatographies of the soluble fraction of *T. molitor* midgut homogenate through HiTrap Phenyl, Resource Phenyl and Resource Q columns showed one activity against the substrate MUC3 (data not shown). It is noteworthy that activity against MUC3 always eluted after the end of the eluting gradient in HIC chromatographies, indicating that this enzyme is

highly hydrophobic. The same is seen with *Serratia marcescen* chitinases (Burberg et al., 1994,1996). Pooled fractions from the last chromatography were named TmChi and SDS-PAGE of this material revealed a single peptide band with 44 kDa (Fig. 1). This agrees with a mass of 40 kDa obtained by gel filtration through a Superdex 75 column (data not shown). A single N-terminal sequence (ATDKIICFFASW) was obtained by microsequencing TmChi, confirming its purity. The recovery and enrichment of chitinase activity during purification are shown in Table 3.

3.3. Properties of TmChi.

TmChi apparently does not have a chitin binding domain (CBD), since only 11 ± 3% (mean plus standard error of mean) binds to colloidal chitin. In the same condition, *S.griseus* and *S. marcescens* chitinases that have CBD bind respectively, 70 ± 2% and 88 ± 1% of their activities to colloidal chitin.

Kinetic parameters of TmChi acting on synthetic substrates are listed in Table 4. Taking k_{cat}/K_M ratios, MUC3 is the best substrate for the enzyme, primarily because of a high velocity constant of hydrolysis. Nevertheless, TmChi shows a strong inhibition by high concentrations of MUC2 or MUC3 (Fig. 2). Due to this inhibition, MUNAG was used as substrate in competition and chemical modification experiments.

Purified TmChi has low activity against colloidal chitin - approximately 1,500 mU/mg, 13% of the activity against MUC3 (12,000 mU/mg). In order to determine the K_M values for chitooligosaccharides (polymerization degrees ranging from 2 to 6), we estimated the inhibition of MUNAG hydrolysis in the presence of different concentrations of the oligosaccharides and a simple linear competitive inhibition pattern was obtained. As we found straight lines in all Lineweaver-Burk plots, this led to the conclusion that only one oligosaccharide molecule binds at each enzyme active site. In these cases, K_i and K_M values are identical. Kinetic parameters of inhibition are displayed at Table 5.

TmChi cleavage pattern of chitooligosaccharides was studied by using capillary electrophoresis of the products generated. At initial rates, TmChi does not hydrolyze chitobiose, and chitotriose. The enzyme forms chitobiose from chitotetraose, and generates chitobiose and chitotriose from chitopentaose with high efficiency. Chitohexaose was hydrolyzed in at least three different linkages, generating chitobiose and chitotriose (Table 5).

TmChi has two ionizable groups involved in catalysis with pK_{E1} = 5.78 ± 0.08 and pK_{E2} = 7.35 ± 0.07 (Fig. 3A). Binding of substrate (MUNAG) significantly affects the dissociation

constants of those groups, as pK_{ES1} = 5.12 ± 0.01 and pK_{ES2} = 7.62 ± 0.02 (Fig. 3B). TmChi does not lose activity with phenylglyoxal, diethylpyrocarbonate, tetranitromethane or EDTA (data not shown), but quickly inactivates at low concentrations of pHMB, EDC or NBS (Fig. 4). TmChi is completely protected against EDC or NBS in the presence of saturating concentrations of the substrate MUNAG, but at this condition inactivation by pHMB is increased (Fig. 4).

3.4. Cloning of the cDNA that codes for TmChi.

During the screening of a cDNA library constructed from *T. molitor* midgut cells, a clone homologous to chitinases from family 18 of glycoside hydrolases was sequenced by chance. Its complete sequence was obtained with a specific primer and shown to code for a protein with the same N-terminal as TmChi (see 3.2). N-terminal amino acid residues are not conserved in family 18 chitinases..

TmChi cDNA has a poly A tail (12 bp), a signal peptide (corresponding to 21 amino acids) and codes for a mature protein with 346 amino acids, with an estimated molecular weight of 37240 Da and an isoeletric point of 4.17 (Fig. 5). This pI value is consistent with TmChi binding to the HiTrap Q and Resource Q anionic exchange columns at pH 7. TmChi amino-acid sequence has three putative glycosylation sites, at residues T268, T269 and N226, the consensus sequence (F/L)DG(F/L/I)D(L/I)DWEYP, with catalytic amino acids D142, D144, Y203 and conserved Cys residues C28, C53, C288, C351 (Fig. 5). TmChi is homologous to the catalytical domain of other insect chitinases as the two chitinases from *Triboleum castaneum* (identities 49% and 46%, GenBank accession numbers AAW67571.1 and AAW67572.1), *Anopheles gambiae* (identity 42%; EAA03527.3), *Apis mellifera* (identity 41%, XP_397146.2) and *Glossina morsitans* (identity 40%, AAL65401.1). The Ser-Thr-rich connector and the chitin binding domain found in other chitinases are lacking in TmChi (Fig. 6), in agreement with the found inability of this enzyme to bind colloidal chitin (see 3.3).

A putative chitinase from *T. molitor* epidermis was described (TmChit5, Royer et al.,2002). It has five units sharing similarities with members of the chitinase 18 family and its first two units apparently have lost their catalytical activity. TmChi shows less identity to TmChit5 domains (less than 31%) than to chitinases from the other insects described above.

4. DISCUSSION

4.1. Occurrence and properties of TmChi.

A 44 kDa chitinase (TmChi) was purified from *T. molitor* midguts. It is not acquired from food, which has a negligible activity upon MUC3. The presence of a poly-A tail in the cDNA sequence that codes TmChi rules out secretion by bacterial microflora. Furthermore, TmChisequence analysis using Target P software(Emanuelson et al., 2000) suggests TmChi is a secretory enzyme. The possibility of chitinase secretion by fungi or protozoa remains, but recent work showed that axenic larvae have chitinase activity similar to control ones (Genta et al., 2006). Thus, the enzyme is probably secreted by midgut epithelial cells.

Attempts to purify TmChi showed that this enzyme is unstable, being digested by midgut proteinases but this is prevented by the proteinase inhibitors E64 plus PMSF. TmChi is probably digested by the cathepsin L-like (Cristofoletti *et al.*, 2005) and serine proteinases from *T. molitor* midgut (Cristofoletti *et al.*, 2001). As much as we are aware this is the first gut chitinase purified from an insect. The enzyme instability and very low activity against colloidal chitin (the most common used chitinase substrate) may be reasons for the lack of data on this kind of enzyme.

TmChi has low activity against colloidal chitin, which is unexpected taking into account the activity observed in *T. molitor* midgut homogenates. The discrepant results may derive from the synergism between TmChi and the highly active N-acetyl-glucosaminidase of the insect, as observed in chitinolytic enzymes from *M. sexta* (Kramer and Koga, 1986). This is supported by the finding that during TmChi purification, separation of chitinase (strong activity against MUC3) from N-acetyl-glucosaminidase (strong activity against MUNAG) resulted in a twofold reduction of chitinase activity against colloidal chitin. Other possibilities are the existence of some minor chitinolytic activity different from TmChi, or the presence of non-catalytical proteins that are essential for degradation of crystalline chitin, as observed in some organisms (Vaaje-Kolstad *et al.*, 2005).

The absence of CBD and of a serine-threonine-rich connector in TmChi might account for the strong susceptibility of TmChi to proteinase attack and its low activity against colloidal chitin. Work done with *M. sexta* chitinase showed that these two domains protect the enzyme against proteolytic attack, and that the CBD is essential for the activity against colloidal chitin (Arakane et al., 2003). It is noteworthy that TmChi and truncated *M. sexta* chitinase share some particular characteristics, as strong substrate inhibition (Arakane *et al.*, 2003).

Substrate inhibition has been described in chitinases from *Serratia marcescens* (Brurberg et al., 1996)**,** *M. sexta* (Zhu *et al*., 2001) and *B. mori* (Koga *et al.*, 1997). Koga *et al*. had suggested that this kind of inhibition shows that oligosaccharides are not the *in vivo* substrate of

the *B. mori* chitinase, but inhibition by high concentrations of polysaccharides as colloidal chitin and CM-chitin-RBV (carboxymethyl–chitin–Remazol Brilliant Violet SR) were observed in *M. sexta* chitinase (Arakane *et al.*, 2003), challenging the functional explanation. Substrate inhibition can be the result of non-productive binding of a second molecule of substrate near or at the active site.

TmChi active site has at least six subsites, since the k_{cat}/K_M ratio reaches a maximum when hexaose is used as substrate. Nevertheless, the knowledge of the specific bonds cleaved in each saccharide is needed to determine subsite distribution and affinity at TmChi active site.

TmChi and other chitinases from family 18 digest oligosaccharides releasing similar products (Sasaki *et al*., 2002). The activity of TmChi increases with substrate size, but generates chitotriose in larger amounts than the other enzymes studied. Due to this wider spectra of products, it is probable that TmChi has an active site more open and larger than those of the other chitinases. Nevertheless, the denomination endo-chitinase is not appropriate for TmChi, because it has low activity against chitin. TmChi definitively is not an N-acetyl-glucosaminidase, because it hydrolyses MUC3 more efficiently than MUNAG, and it is completely inactive against chitobiose. Thus, no appropriate description for TmChi exists in the enzyme classification system of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/).

TmChi pH-activity profiles are bell shaped, as the one obtained for the human enzyme (Chou et al.,2006). The same is not true for *Manduca sexta* chitinase, which pH-activity profile is broad and its slope appears to be determined by multiple ionisable groups (Lu et al.,2002). In *Serratia marcescens* chitinase B, only one ionisable group is detected, with a pKa value near 7.0 (Synstad et al.,2004). It seems that catalysis in family 18 chitinases are mediated by several amino acid residues (Synstad et al.,2004; Lu et al.,2002) and it is not evident which groups are responsible for the pH-activity profile.

Inactivation data by EDC support a role for carboxylates in TmChi catalysis, and four active site carboxylates are strongly conserved in family 18 chitinases, which participation in catalysis has been established by mutagenesis and crystallographic studies (van Aalten *et al.*, 2001). In TmChi, these residues are Asp 142, Asp 144, Glu 146 and Asp 204. The basic side of the pH-activity profile of chitinases from family 18 is not determined by deprotonation of the catalytic proton donor (E146), but rather by deprotonation of D144. Upon substrate binding, protonated D144 rotates and shares a proton with E146. This stabilizes deprotonated E146 after protonating the glycosidic oxygen of the susceptible substrate bond (van Aalten *et al.*,

2001). The role proposed for D144 agrees with the observed increase of pK_2 after substrate binding, because a hydrogen bond between D144 and E146 raises the pKa of the first.

The acidic part of the curve may depend on the titration of E146 that is required for enzymatic activity in *M.sexta* chitinase, which has a pH-activity profile more similar to TmChi (lu et al.,2002)

The pHMB-modified Cys residue that is important for TmChi activity is near the active site, because binding of MUNAG leads to its exposure. This Cys residue should be located at a subsite other than +1/-1 subsites, and may be the residue C129. The other Cys residues of TmChi (C28, C53, C288, C351) are fully conserved in other animal family 18 proteins and are involved in the disulfide bonds C28-C53 and C288-C351 (Fusetti *et al.*, 2003).

NBS inactivates TmChi quickly at very low concentrations, and the enzyme is protected from inactivation by the small molecule of MUNAG. These facts suggest the involvement of an exposed Trp residue in substrate binding. The exposure of such hydrophobic group may partly explain the high hydrophobicity of TmChi that is observed when this protein is subjected to HIC chromatography. Inactivation protection conferred by MUNAG suggests that this Trp is very near the cleavage site, at subsites -1 or +1. Trp residues are likely to be involved in planar packing with the sugar ring of the substrate (van Aalten, 2001; Fusetti et al., 3003). Several Trp residues are conserved in the catalytic domain of family 18 chitinases. In TmChi these residues are W33, W105, W145, W294 and W340. Based on crystallographic assignment, Trp residues at cleavage site are W105 (subsite +1) and W340 (subsite -1) (van Aalten, 2001). In this way, both can be the TmChi NBS-modified residue essential for substrate binding and which is protected by MUNAG.

4.2. Role of TmChi.

Three functions had been proposed for insect midgut chitinases: (1) intermolt peritrophic membrane (PM) digestion (Bolognesi et al., 2005) (2) controlling peritrophic membrane (PM) width (Shen and Jacobs-Lorena, 1997) and (3) digestion of chitin-rich structures (Souza-Neto et al., 2003). In the first two cases, gut chitinase activity is part of a very sensitive equilibrium between synthesis and degradation of PM, leading to formation, maintenance and degradation of this structure. Any abrupt change in chitinase activity leads to the disturbance of insect digestive function, reinforcing the proposal of chitinase as a target for insect control (Kramer and Muthukrishnan, 1997).

To digest chitin-rich structures present in the diet, chitinase has to be secreted in high amounts. This may be the case of insect-feeding insects, but is also important for complete fungal cell wall disruption in detritus-feeding insects. Fungal cell disruption is necessary to make available the nutritive cell contents (Terra and Ferreira, 1994, 2005).

T. molitor food is rich in fungal cells that are almost absent from the midgut (Genta et al., 2006). Recently, a laminarinase with high lytic power against fungal cell walls was purified and characterized from *T. molitor* midgut (Genta, F.A., Terra, W.R., Ferreira, C., manuscript in preparation). The expression of *T. molitor* laminarinase, β-glucosidase and chitinase seem to have a concerted regulation (Genta et al, 2006).Thus, it is possible that TmChi has an accessory role in fungal cell wall breakdown, complementing the action of laminarinase and β-glucosidase. Another TmChi role may be chitooligosaccharide digestion, to release monosaccharides, thus taking full advantage of the nutritionally poor diet.

To perform digestive functions a highly expressed midgut chitinase is necessary. Nevertheless, the presence in the midgut of high amounts of a chitinase with a CBD might be deleterious to the insect by PM damage (Kramer and Muthukrishnan, 1997). This probably led to the selection of a truncated form of the gene lacking CBD. The necessity of an enzyme to have chitin binding activity to damage PM is also illustrated by an interesting observation. Some plants respond to insect attack by producing a chitin-binding cysteine proteinase that damages PM (Pechan *et al.*, 2002). Some characteristics of TmChi (specially the absence of CBD) may be overspread among detritus-feeding insect midgut chitinases. *Triboleum castaneum*, a species related to *Tenebrio molitor,* has 3 chitinase sequences (Chi3, GenBank accession number AAW67570.1; Chi4, AAW67571.1; Chi5, AAW67572.1) that have no CBD and may function as TmChi. It is noteworthy that the midgut chitinase from the detritivorous *Chironomus* larvae is also truncated (see Fig. 6), and that midgut chitinase activities in *A. aegypti* larvae have molecular weights ranging between 30-40 kDa (Souza-Neto *et al.*, 2003), indicating that they may lack the CBD. The truncated enzyme from the phytophagous beetle *P. cochleariae* disagrees with this hypothesis, but it is probably a conserved characteristic from the detritus-feeding coleopteran ancestor (Terra and Ferreira, 1989).

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Legends of Figures

Fig. 1. Proteins resolved by electrophoresis in SDS–12% polyacrylamide gel slabs. Lane 1, molecular mass standards; 2, soluble fraction of midgut luminal contents; 3, active fractions upon MUC3 pooled after HiTrap Phenyl hydrophobic interaction chromatography; 4, chitinase active fractions eluted from Resource Phenyl hydrophobic interaction chromatography; 5, Chitinase active fractions eluted from Resource Q ion exchange chromatography, and 6, chitinase active fractions eluted from second Resource Q ion exchange chromatography.

Fig. 2. Effect of the concentration of different substrates in the activity of purified chitinase (TmChi). Note inhibition by excess substrate when activity against MUC2 (□) or MUC3 (△) are measured.

Fig. 3. Effect of pH on the kinetic parameters of TmChi with MUNAG as substrate. The points are experimental and the curves are theoretical based on the constants (found by least-squares method) described in each case. (A) Effect of pH on $Vmax_{app}/ Km$ ratio. pK_{E1} = 5.78 ± 0.08 and pK_{E2} = 7.35 ± 0.07 (B) Effect of pH on $Vmax_{app}$. pK_{ES1} = 5.12 ± 0.01 and pK_{ES2} = 7.62 ± 0.02. TmChi is stable during the assay time in the range pH 2-10.

Fig. 4 – Chemical inactivation of TmChi. (A) Inactivation with EDC with or without 10 mM MUNAG. (B) Inactivation with NBS with or without 10 mM MUNAG. (C) Inactivation with pHMB with or without 10 mM MUNAG.

Fig. 5. - Nucleotide and deduced amino acid sequences of *T. molitor* chitinase (TmChi). The stop codon and poly A tail are in bold. The arrow indicates the signal peptide cleavage point, deduced from the N-terminal amino acid sequence of purified TmChi. Amino acids identical to those microsequenced from the purified TmChi are underlined. Residues conserved in all family 18 insect chitinases are shaded, residues with black background are directly involved in catalysis, and glycosylation sites are boxed. The sequence was deposited in the GenBank™ under the ID AAP9218.1.

Fig. 6. - Amino acid sequence alignment of insect chitinases from the glycoside hydrolase family 18. The sequences were retrieved from GenBank™. The listed chitinases are from *Bombyx mori* (AAA47538.1); *Manduca sexta* (AAB53952.1); *Helicoperva armigera* (AAQ91786.1); *Aedes aegipty* (AAB81849.1); *Anopheles gambiae* (AAB87764.1); *Glossina morsitans* (AAL65401.1); *Chironomus tentans* (CAA73685.1); *Chelonus sp*. (AAA61639.1); *Tenebrio molitor* TmChi (AAP9218.1) and *Phaedon cochleriae* (CAA77014.1). Strongly

conserved residues are shaded. *M. sexta* chitinase serine-threonine-rich connector (residues 397-497) and chitin-binding domain (residues 498-555) are double underlined and boxed, respectively.

Table 1 – Activity against colloidal chitin in the gut of insects from three different orders.

Insect	mU/midgut	mU/g tissue	mU/mg protein
S. frugiperda (Lepidoptera)	2.4±0.8	10±3	3±1
P. americana (Dictyoptera)	8±3	60±30	3±1
T. molitor (Coleoptera)	11±2	600±100	14±4

Results are means and SEM from three determinations. mU/midgut are the amount of chitinase mUnits present in each insect midgut. Fresh weights of *Spodoptera frugiperda*, *Periplaneta americana* and *Tenebrio molitor* entire guts are 250 ± 10 mg, 150 ± 5 mg e 20 ± 2 mg, respectively.

Table 2 - Hydrolases and protein present in different gut sites of *T. molitor* larvae.

Substrate	Midgut				Hindgut
	Anterior		Posterior		
	contents	cells	contents	cells	
C. Chitin	71 (7)	10 (1)	10 (2)	8 (2)	1 (0.4)
p-NAG	60 (2600)	8 (400)	28 (2000)	4 (300)	0.2 (30)
MUC2	66 (67)	6 (7)	23 (36)	5 (10)	0.5 (0.9)
MUC3	60 (32)	8 (4)	26 (22)	5 (5)	1 (1.4)
Protein, μg	390	330	220	190	170

Enzyme results are relative activities displayed as percentage of the sum of activities found in the different sections of the gut and specific activities (in parentheses) displayed as mU/mg protein. Figures are means based on determinations carried out in five different preparations obtained from five insects each. SEM were found to be 5–20% of the means. C. Chitin, colloidal chitin.

Table 3 – Purification of chitinase from *T. molitor* larval midgut.

Fraction	Specific activity mU/mg	Yield (%)	Purification factor
Soluble fraction of midgut	90	100	1
HiTrap Phenyl eluate	570	125	7
Resource Phenyl eluate	5000	30	57
First Resource Q eluate	6300	30	72
Second Resource Q eluate	11900	11	135

Substrate used: MUC3

Table 4 - Kinetic parameters of TmChi acting on synthetic substrates

Substrate	k_{cat} (s^{-1})	K_m (mM)	K_i (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat}/K_m, relative
MUNAG	300±10	0.05±0.01	N.D.	6000±1000	10
MUC2	350±40	0.014±0.006	0.031±0.005	25000±10000	43
MUC3	1500±200	0.026±0.005	0.035±0.008	60000±10000	100

MUNAG, methylumbelliferyl-β-N-acetylglucosamine; K_i, constant of inhibition by excess substrate; N.D., not detected.

Table 5 – Products released by TmChi from chito-oligosaccharides (MUNAG as substrate) and cleavage of these saccharides by the enzyme.

Saccharide	K_i, **mM**	**Products**	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Chitobiose (C2)	0.04 ± 0.01	N.D.	N.D.
Chitotriose (C3)	0.13 ± 0.01	ND	ND
Chitotetraose (C4)	0.98 ± 0.03	C2	105 ± 7
Chitopentaose (C5)	0.023 ± 0.004	C2+C3	128 ± 9
Chitohexaose (C6)	0.18 ± 0.01	C2	100 ± 10
		C3	90 ± 10

K_i, dissociation constant of the oligossacharide when inhibiting MUNAG hydrolysis; N.D., not detected.

Figure 1:

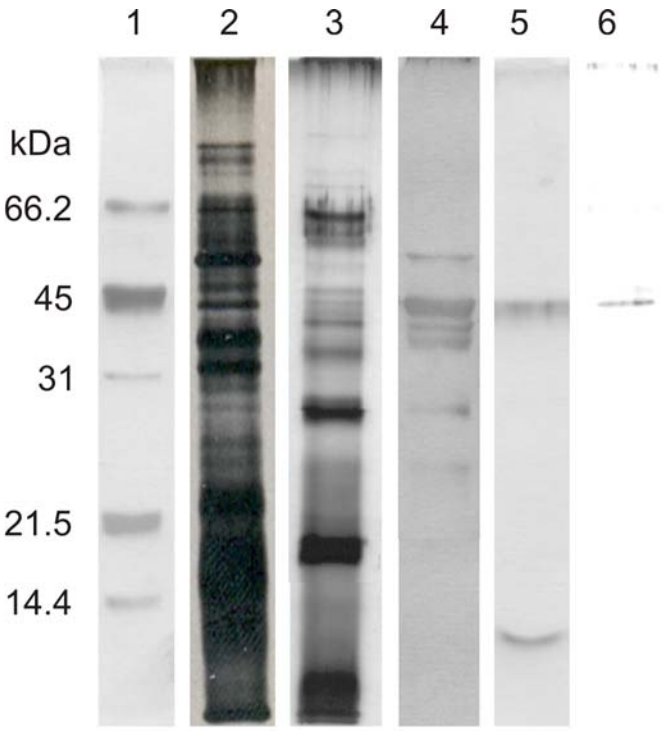


Fig. 1

Figure 2:

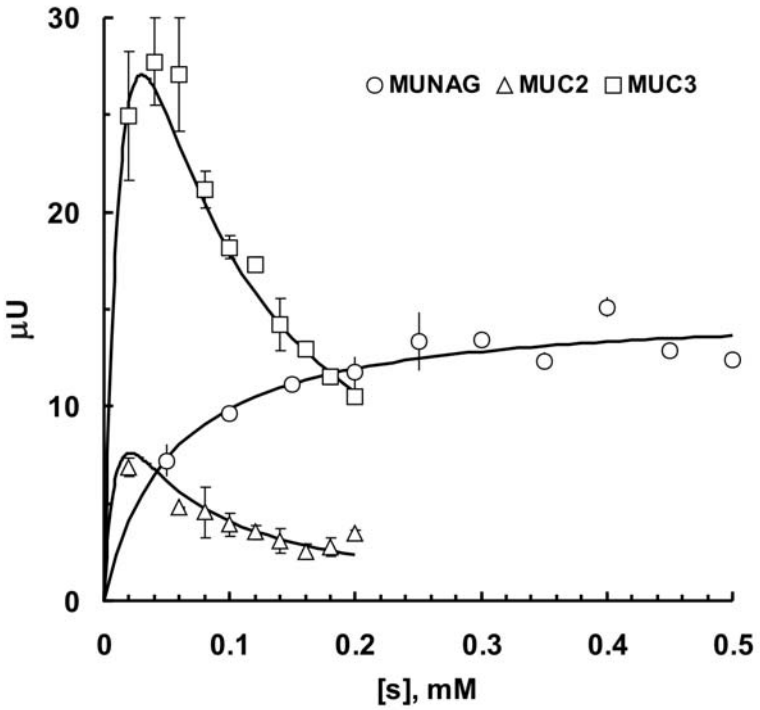


Figure 3:

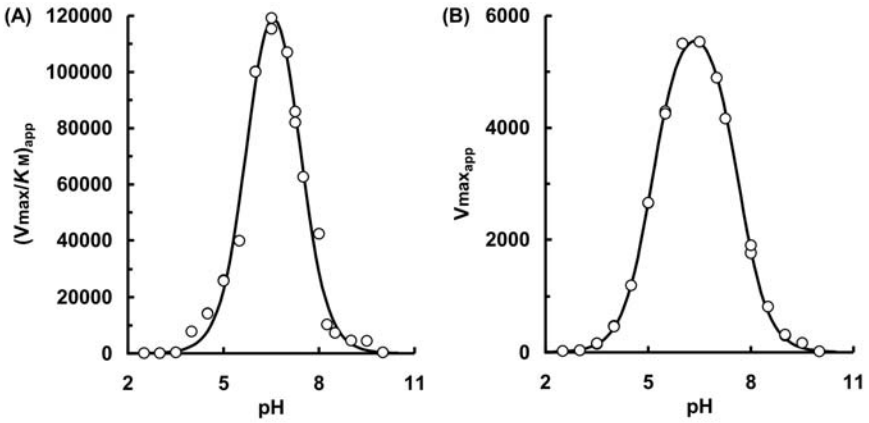


Fig. 3

Figure 4:

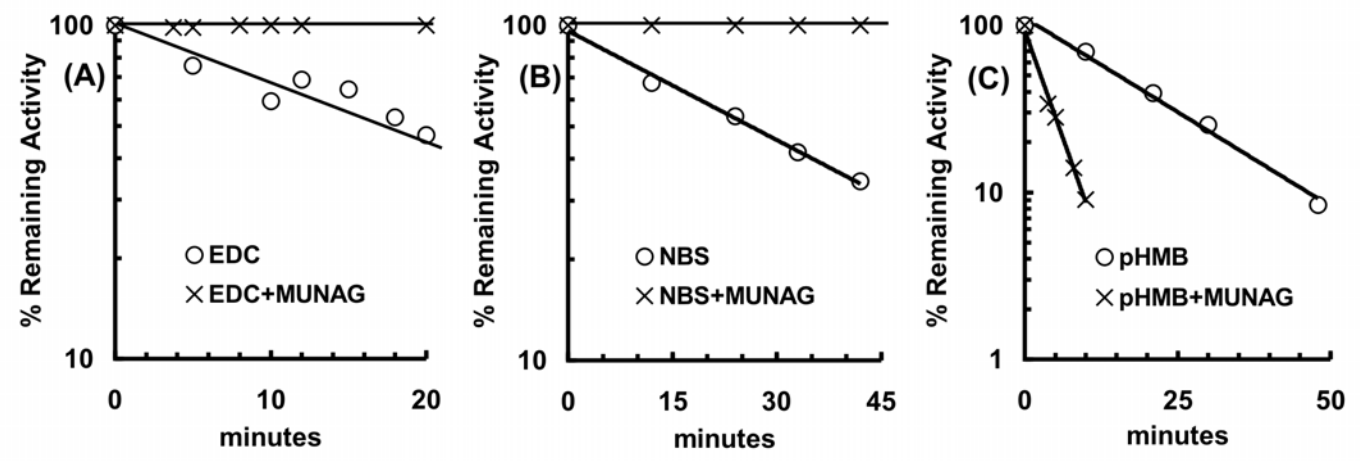
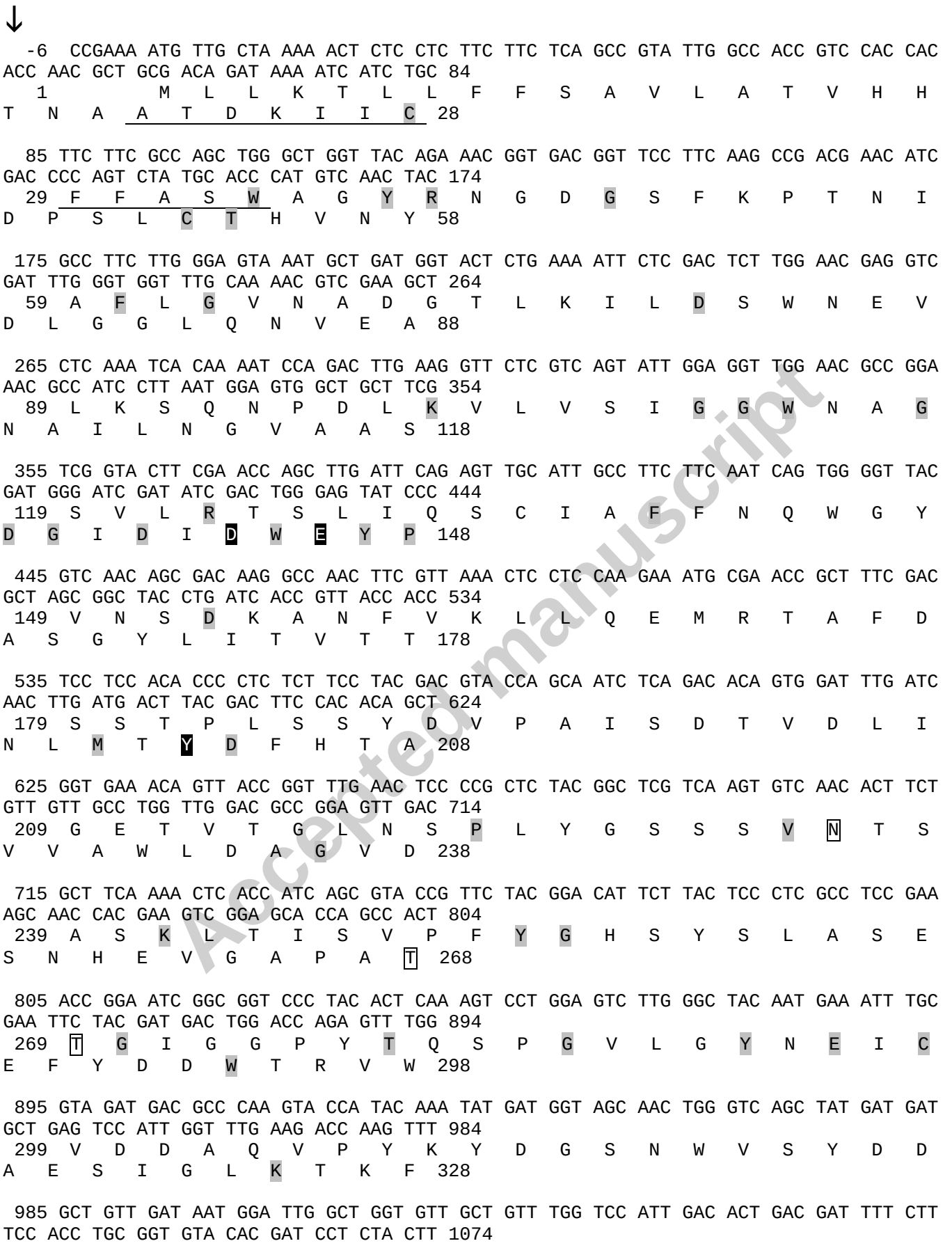


Figure 5:



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 329  A   V   D   N   G   L   A   G   V   A   V   W   S   I   D   T   D   D   F   L
S   T   C   G   V   H   D   P   L   L  358

1075 CAA GCC ATC AAA GAC AAC CTT TCG GCT TAG
ATAAGATGATTTCTTAAAAATCTTGTAACCACGTGTTTCATTAAATGTTTTAAATAAAAAAAAAAAA 1171
 359  Q   A   I   K   D   N   L   S   A   *
367
```

Fig. 5

Figure 6:

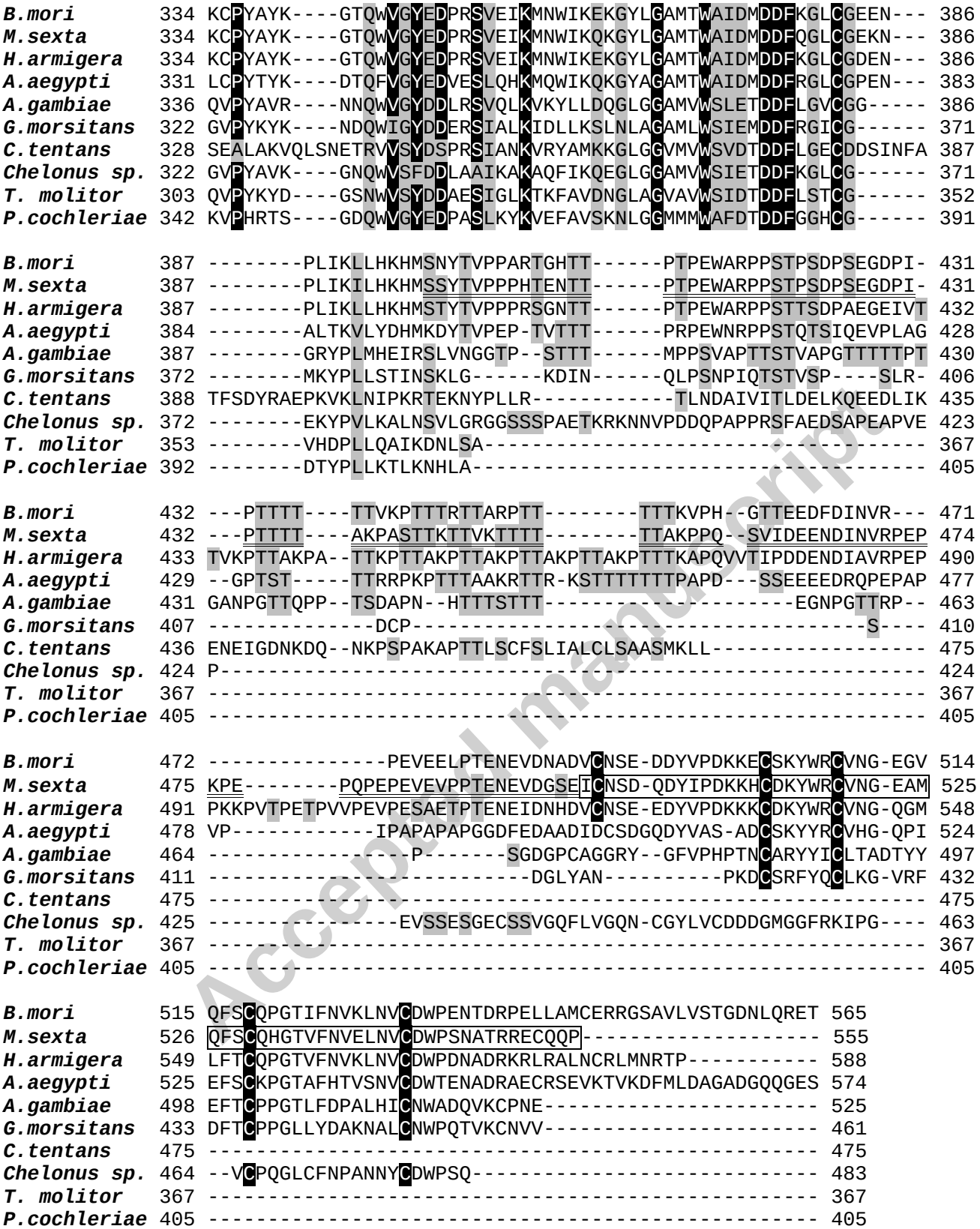

Fig.6