

Cloning, purification and comparative characterization of two digestive lysozymes from *Musca domestica* larvae

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cDNA coding for two digestive lysozymes (Mdl1 and Mdl2) of the *Musca domestica* housefly was cloned and sequenced. Mdl2 is a novel minor lysozyme, whereas Mdl1 is the major lysozyme thus far purified from *M. domestica* midgut. Mdl1 and Mdl2 were expressed as recombinant proteins in *Pichia pastoris*, purified and characterized. The lytic activities of Mdl1 and Mdl2 upon *Micrococcus lysodeikticus* have an acidic pH optimum (4.8) at low ionic strength ($\mu = 0.02$), which shifts towards an even more acidic value, pH 3.8, at a high ionic strength ($\mu = 0.2$). However, the pH optimum of their activities upon 4-methylumbelliferyl N-acetylchitotrioside (4.9) is not affected by ionic strength. These results suggest that the acidic pH optimum is an intrinsic property of Mdl1 and Mdl2, whereas pH optimum shifts are an effect of the ionic strength on the negatively charged bacterial wall. Mdl2 affinity for bacterial cell wall is lower than that of Mdl1. Differences in isoelectric point (*pI*) indicate that Mdl2 (*pI* = 6.7) is less positively charged than Mdl1 (*pI* = 7.7) at their pH optima, which suggests that electrostatic interactions might be involved in substrate binding. In agreement with that finding, Mdl1 and Mdl2 affinities for bacterial cell wall decrease as ionic strength increases.

Key words: Lysozyme; Digestive lysozyme; Substrate affinity; pH optimum

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Introduction

Lysozymes (EC 3.2.1.17) catalyze the hydrolysis of β -1,4 glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid residues in the peptidoglycan of the bacterial cell wall. These enzymes are classified in the glycoside hydrolase families 22, 23, 24, 25, and 73 (1).

Of the c-type lysozymes, found in family 22, the hen egg white lysozyme (HEWL) is the most extensively studied. This lysozyme has 129 amino acid residues that form an ellipsoidal structure containing two domains. One of

them (residues 40 to 85) is rich in β -sheets, whereas the other (residues 1 to 39, and 101 to 129) contains α -helices (2). The active site, a deep cleft between these two domains, is divided into six subsites, each of them interacting with one monosaccharide unit and originally called A to F. Substrate cleavage occurs between sites D and E (3,4). However, according to the current nomenclature for subsites in glycoside hydrolases (5), subsite A should be called -4, and subsite F, +2. The HEWL catalytic mechanism has two steps: the first, called glycosylation, results in the formation of a glycosyl-enzyme intermediate, whereas

the second is the hydrolysis of that intermediate (6). In this reaction, carboxylic groups of the side chain of E35 and D52 (HEWL numbering) act as a catalytic acid/base and a nucleophile, respectively.

Lysozymes from family 22 are usually involved in the defense against bacteria. However, ruminant artiodactyls that have a bacterial culture in their foregut use lysozymes to cleave the walls of those bacteria and release their nutrients (7). Leaf-eating birds and monkeys also have digestive lysozymes (7-9). Insects that feed on bacteria growing in decomposing material (Diptera such as the housefly *Musca domestica* and the fruit fly *Drosophila melanogaster*) also have digestive lysozymes, which take part in the disruption of bacteria in their diet (10-12). The catalytic properties of the digestive lysozymes from *M. domestica* (MdL1) and *D. melanogaster* (LysD) were studied by Lemos et al. (13) and Regel et al. (12), whereas the spatial structure of MdL1 was only recently determined (14).

Comparisons between digestive and non-digestive lysozymes revealed adaptive properties related to the digestive function, such as a low isoelectric point (pI), resistance to proteolytic enzymes, acidic pH optimum, reduction in the number of acid-labile peptide bonds, and decrease in the number of arginine residues (15). Some of these properties are easily associated with changes in the primary sequence of these proteins. However, properties such as resistance to proteinases and acidic pH optimum demand more complex explanations. For instance, the structural comparison between HEWL and MdL1 revealed that, in spite of the overall similarity between the folding of these two lysozymes, changes of the amino acid residues near the catalytic residues (E32 and D50 in MdL1) determined the acidic pH optimum of the digestive lysozymes upon synthetic substrates, such as 4-methylumbelliferyl N-acetylchitotrioside (MUNAG3) (14). Nevertheless, the basis of the bacteriolytic activity, which includes acidic pH optimum and decreases at high ionic strengths, remains to be fully understood. The answers to these questions depend on the characterization of several digestive lysozymes on the basis of a common set of enzyme kinetic experiments, which will facilitate the comparison of digestive and non-digestive enzymes.

The present study reports on the molecular cloning, sequencing, expression as recombinant protein, and purification of a new digestive lysozyme from *M. domestica* (MdL2). It also describes the catalytic properties (pH optimum and substrate affinity) of MdL2 and compares them with those of MdL1. Finally, based on these data, an explanatory hypothesis for their shared catalytic properties is proposed.

Material and Methods

Animals

Larvae of *M. domestica* were reared in a mixture of fermented commercial pig food and rice hull (1:2; v/v) at 24°C and constant light. Third-instar larvae were fed starch one day before dissection. Only actively feeding larvae were used in this study.

Molecular cloning of MdL1 and MdL2

Total RNA from midgut epithelium of 119 larvae of *M. domestica* was extracted using Trizol (Life Technologies, USA) according to manufacturer instructions. To obtain MdL1 and MdL2 nucleotide sequences, a rapid amplification of cDNA end strategy was used (16) (Figure 1). The cDNAs corresponding to the total RNA pool from the midgut epithelium of *M. domestica* larvae were synthesized using reverse transcription. This sample was used as a template, and the 5' and 3' ends of the cDNA coding for MdL1 were amplified using primers directed to a conserved region of dipteran lysozymes and to their 5' and 3' ends (Figure 1). The cDNA coding for the complete sequence of the mature MdL2 was amplified following the same strategy, but using primers directed to the N- and C-terminal sequence of dipteran lysozymes (Figure 1). These samples of amplification products were cloned separately and multiplied in bacteria, and, finally, the recombinant plasmids were sequenced individually.

On the basis of these results, specific primers for the ends of MdL1 were designed (LysF: 5' CGC GGA TCC AAA CGA TGA AAT TCT TCA TTG TCT TG 3'; LysR: 5' CCC AAG CTT GAA TTC TTA AAA ACA GTC GTT GAT GCT G 3') and used to amplify the complete sequence of the lysozyme with the cDNA prepared initially as a template. This amplification product was cloned into a pGemT-Easy vector (Promega Corp., USA) and sequenced.

Expression of MdL1 and MdL2 in *Pichia pastoris*

A pGemT-Easy vector containing an insert that codes for MdL1 and Lys-Xho-F (5' CCG CTC GAG AAA AGA GAG GCT GAA GCT AAG ACT TTC ACT CGC TGC 3') and LysR primers (see above) were used to amplify the DNA segment coding for mature MdL1. This product (about 350 bp) was recovered from an agarose gel (1%; w/v) and digested with *Xho*I and *Eco*RI (New England Biolabs, USA) following manufacturer instructions. The digested product was then cloned into the pPic9kf1 vector (Invitrogen, USA) (also digested with *Xho*I and *Eco*RI). Simultaneously, a pGemT-Easy vector containing an insert that codes for the mature MdL2 was digested with *Xho*I and *Eco*RI. The digestion product corresponding to the insert

(about 350 bp) was recovered from an agarose gel (1% w/v) and ligated into a pPic9kf1 vector digested with *Xho*I and *Eco*RI.

After that, pPic9kf1 vectors coding for MdL1 and MdL2 were digested separately using *Sac*I and then used to transform the yeast *Pichia pastoris* (GS115 cells) through electroporation. Transformed colonies, which grew in minimal dextrose medium agar, were detected by DNA polymerase chain reaction (PCR) using the same primers initially used to amplify the MdL1 and MdL2 coding regions.

The production of the recombinant MdL1 and MdL2 was based on the procedure described by Cançado et al. (14). Single colonies of *P. pastoris* transformed with pPic9kf1 coding for MdL1 or MdL2 were grown separately in yeast extract peptone dextrose medium at 160 rpm and 28°C for 18 h. The cultures were then centrifuged at 2500 *g* and 4°C for 5 min. The cells were collected and rinsed three times with autoclaved water. They were then resuspended in buffered minimal methanol medium containing

1% methanol (v/v) and incubated at 160 rpm and 28°C for 72 h. Methanol (final concentration: 1%) was added to the culture every 24 h. Finally, the cultures were centrifuged at 2500 *g* and 4°C for 5 min, the cells were discarded, and supernatants were stored at -20°C.

Purification of the recombinant MdL1 and MdL2

The purification of MdL1 was based on the procedure described by Cançado et al. (14). Briefly, ammonium sulfate (0.652 g/mL) was added to the culture supernatant containing MdL1 and incubated at 25°C for 18 h. The mixture was subsequently centrifuged at 10,000 *g* and 4°C for 1 h. The pellet was resuspended in 20 mM sodium acetate, pH 5.5, and dialyzed against this same buffer at 4°C for 18 h.

A similar procedure was used to precipitate MdL2 from the culture supernatant, but the final pellet was resuspended and dialyzed against 20 mM triethanolamine, pH 8.0. This sample was loaded onto a HighQ column (Bio-Rad, USA). Proteins were eluted with a linear NaCl gradi-

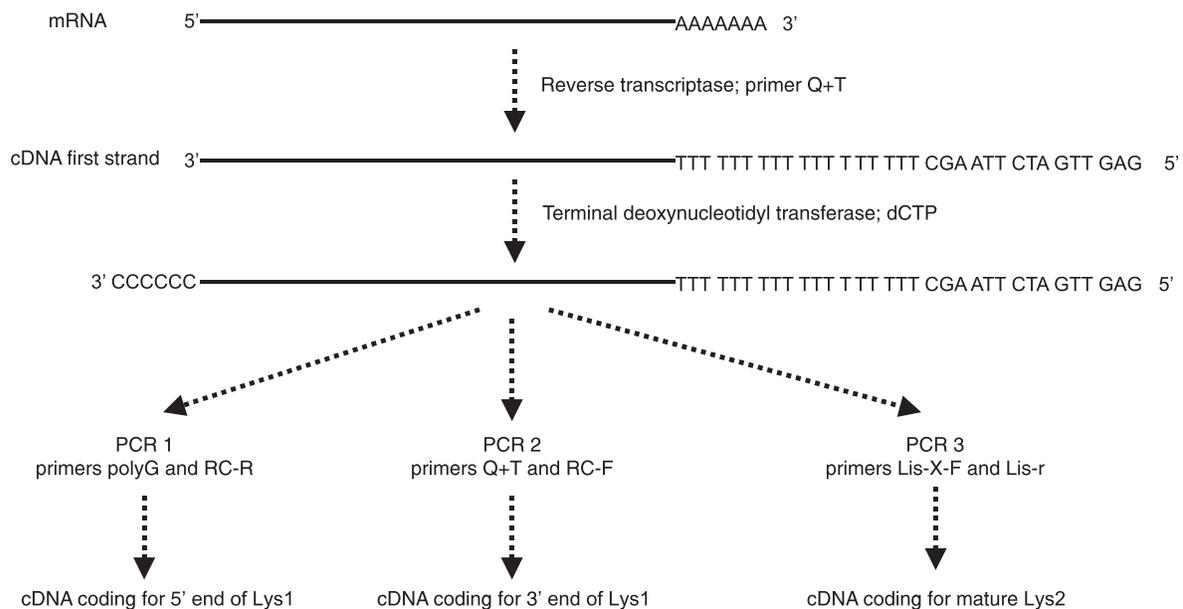


Figure 1. Strategy used to clone cDNA coding for MdL1 and MdL2. cDNA first strand was synthesized using the Superscript First Strand kit (Invitrogen) and a primer directed to mRNA polyA tail (Q+T: 5' GAC TTG ATC TTA TTT TTT TTT TTT TTT TTT 3'). After that, a polyC tail was added to cDNA 3' end using terminal deoxynucleotidyl transferase (TdT) enzyme (Invitrogen) according to reaction conditions described in Ref. 29. The 5' cDNA end of MdL1 was amplified using PCR, cDNA first strand as template, and polyG and RC-R (5' TTG ATC TGG AAG ATT CCG TAG TC 3') primers. The 3' cDNA end of MdL1 was amplified following the same procedure, but using Q+T and RC-F (5' GAC TAC GGA ATC TTC CAG ATC AA 3') primers. RC-R and RC-F primers were designed based on a conserved sequence (DYGIFQIN) found in lysozymes from *Drosophila melanogaster* (30,31), *Musca domestica* (24) and *Anopheles darlingi* (32). cDNA coding for mature MdL2 was amplified using Lis-X-F (5' CTA TAT CCG CTC GAG AAA AGA GAG GCT GAA GCT CGT TGC TCC CTG GCC CGC G) and Lis-r (5' TCA TAG TTT AGC GGC CGC GCA TGC TTA GAA GCA GTC ATC GAT GGA CGG C) primers. Lis-X-F and Lis-r primers were designed on the basis of the N- and C-terminal sequence of *M. domestica* lysozyme (24). Amplification products were cloned into pGemT-Easy vector (Promega) and sequenced.

ent (0 to 1 M; 100 mL; flow rate: 5 mL/min) and the fractions (3 mL) containing lysozyme activity were pooled and dialyzed against 20 mM triethanolamine, pH 8.5. This sample was then loaded onto a MonoQ column (GE Healthcare, USA) and proteins were eluted with a linear NaCl gradient (0 to 1 M; 25 mL; flow rate: 1 mL/min). Fractions (0.5 mL) containing lysozyme activity were pooled and dialyzed against 20 mM triethanolamine, pH 8.5. The purification of MdL1 and MdL2 was verified using polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

Samples containing approximately 4 µg MdL1 and MdL2 were diluted in 120 mM Tris-HCl, pH 6.8, buffer containing 5% SDS (w/v), 1.3 mM β-mercaptoethanol, 1.0 mM EDTA, 20% glycerol (v/v) and 0.01% bromophenol blue (w/v). These samples were heated for 5 min at 95°C and loaded onto 14% polyacrylamide gel containing 1% SDS (w/v) (17). Electrophoresis was performed at a constant potential (200 V). Proteins were stained using silver nitrate (18). The relative molecular weights of MdL1 and MdL2 were calculated as described by Shapiro et al. (19) using the following standards (BioRad): HEWL, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31.0 kDa; ovalbumin, 45 kDa; albumin, 66.2 kDa, and phosphorylase b, 97.4 kDa.

Protein determination

Protein concentration was determined using Coomassie brilliant blue G according to Bradford (20) and ovalbumin as the standard.

Lysozyme assays

Lysozyme activity was determined measuring the turbidity decrease at 650 nm of a *Micrococcus lysodeikticus* suspension (1 mg/mL) prepared in 100 mM citrate-sodium phosphate, pH 3.5, buffer containing 150 mM NaCl (except as otherwise specified). The final volume of the reactions was 0.25 mL. The enzymatic reaction was terminated by the addition of 0.5 mL 0.5 M sodium carbonate. These assays were performed at 30°C. Only assays with a linear relationship between the decrease of turbidity and the incubation time were used. One lysozyme activity unit was defined as the amount of enzyme that causes a decrease of 0.01 absorbance units at 650 nm/min.

Lysozyme activity upon MUNAG3 was determined by measuring the release of the methylumbelliferone fluorescent group (excitation at 360 nm, emission at 450 nm). The substrate was prepared in water (initial concentration of 0.15 mM) and then diluted with the appropriate buffer, resulting in a final concentration of 7.8 µM. These enzy-

matic reactions were performed at 30°C, in a final volume of 0.2 mL, and were stopped by the addition of 1.0 mL 100 mM glycine-NH₄OH, pH 10.5. The rate of hydrolysis of MUNAG3 was calculated on the amount of product released after four different incubation times. Only assays with a linear relationship between fluorescence and incubation time were used.

Characterization of MdL1 and MdL2

Effect of pH and ionic strength. The effects of pH and ionic strength (µ) on the lytic activities of MdL1 and MdL2 were determined using the following buffers: glycine-HCl (pH 2.0-3.5), sodium acetate (pH 4.0-5.5) and sodium phosphate (pH 6.0-7.5). Buffers were prepared with ionic strengths that ranged from 0.02 to 0.2 (21). The initial rate of the lytic reaction was measured using a constant *M. lysodeikticus* concentration (1 mg/mL).

The effects of pH and ionic strength on MdL1 and MdL2 hydrolysis of MUNAG3 (7.8 µM) were determined in the same pH range described above. However, 100 mM citrate-sodium phosphate buffers were used. NaCl was added to these buffers to maintain the ionic strength at µ = 0.25 or 0.07 regardless of pH. Briefly, the concentration of each ionic compound in the buffer was calculated considering the pH and their pK_a. After that, the ionic strength of the buffer was calculated using the equation $\mu = 1/2 \sum Mz^2$, where M is the molar concentration and z is the electrical charge for each ion from the buffer. Finally, NaCl was added to reach the desired ionic strength.

The stability of MdL1 and MdL2 was determined at pH values ranging from 3 to 7.3. Purified enzymes were incubated in 100 mM citrate-sodium phosphate buffers for a period of time equal to that used in the enzymatic assays (15 min). After that, the remaining activity was determined using a suspension of *M. lysodeikticus* (1 mg/mL) prepared in 200 mM citrate-sodium phosphate, pH 4. All pH measurements were performed at 30°C.

Effect of substrate concentration

The effects of *M. lysodeikticus* concentration on MdL1 and MdL2 activities were determined at the pH optimum and different ionic strengths (0.02, 0.07, 0.1, and 0.2). The initial rates of the lytic reaction at ten different substrate concentrations (0.1 to 2 mg/mL) were used to determine the kinetic parameters (V_{max} and K_m). The data were fitted to the Michaelis-Menten equation using the Enzfitter software (Elsevier-Biosoft, UK).

Sequence analysis

MdL1 and MdL2 translated protein sequences were analyzed using the ProtoParam tool available at Expasy

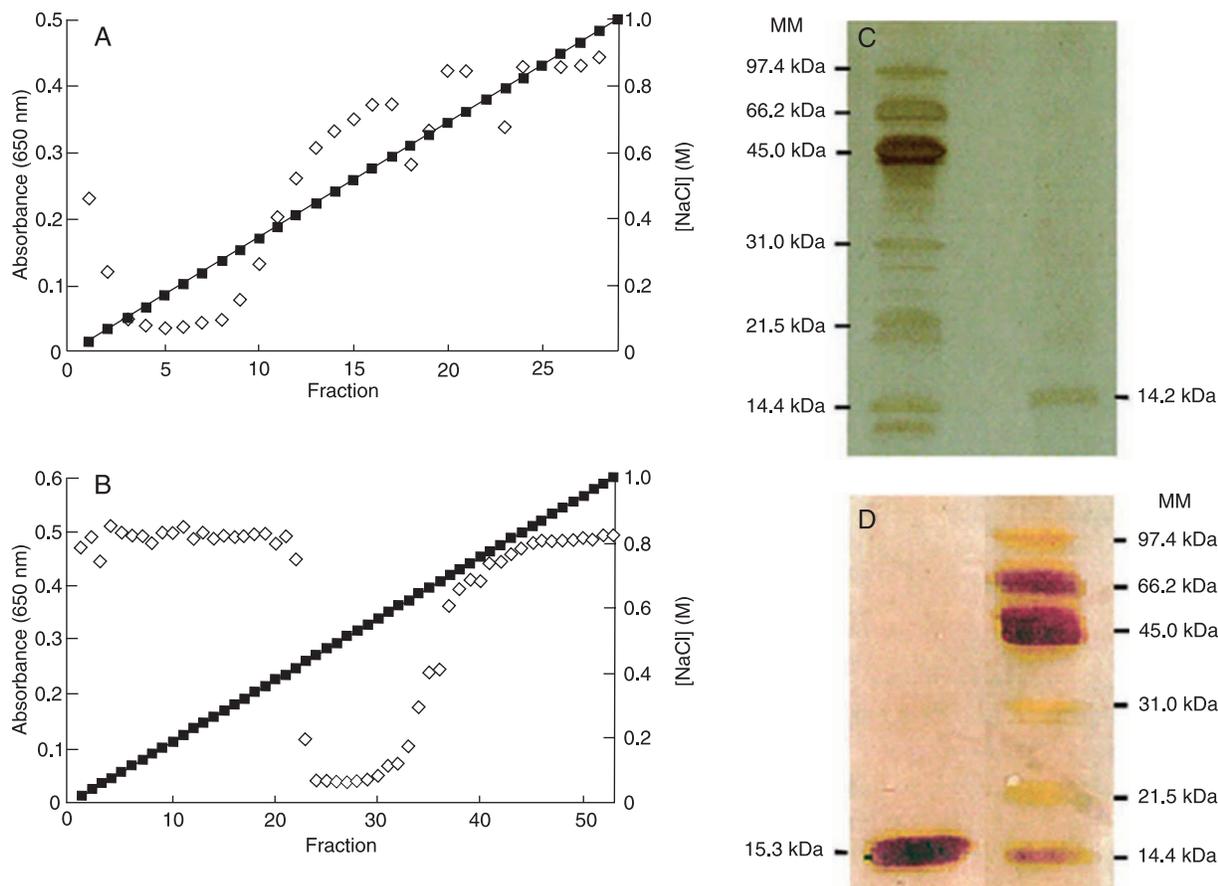


Figure 3. Purification of recombinant Mdl1 and Mdl2. *A*, Material containing Mdl2 resuspended in 20 mM triethanolamine, pH 8.0, after $(\text{NH}_4)_2\text{SO}_4$ precipitation was loaded onto a HighQ column. Proteins were separated by hydrophobic chromatography and eluted with a linear gradient of NaCl (0 to 1 M; filled squares) in the same buffer. Lysozyme activity (open lozenges) was detected by measuring decrease in turbidity of a *Micrococcus lysodeikticus* suspension (1 mg/mL). Fractions with lysozyme activity were pooled and dialyzed against 20 mM triethanolamine, pH 8.5. *B*, Pooled fractions obtained in panel *A* were loaded onto a MonoQ column. Proteins were eluted as described in *A*. *C* and *D*, SDS-PAGE of purified Mdl1 (*C*) and Mdl2 (*D*). As described in Material and Methods, after $(\text{NH}_4)_2\text{SO}_4$ precipitation a sample containing 4 μg Mdl1 was loaded on the gel marked *C*. Fractions 23 to 36 obtained in panel *B* were pooled and a sample containing 4 μg protein was loaded on the gel marked *D*. Polyacrylamide gels (14%) were silver stained. MM, molecular weight markers.

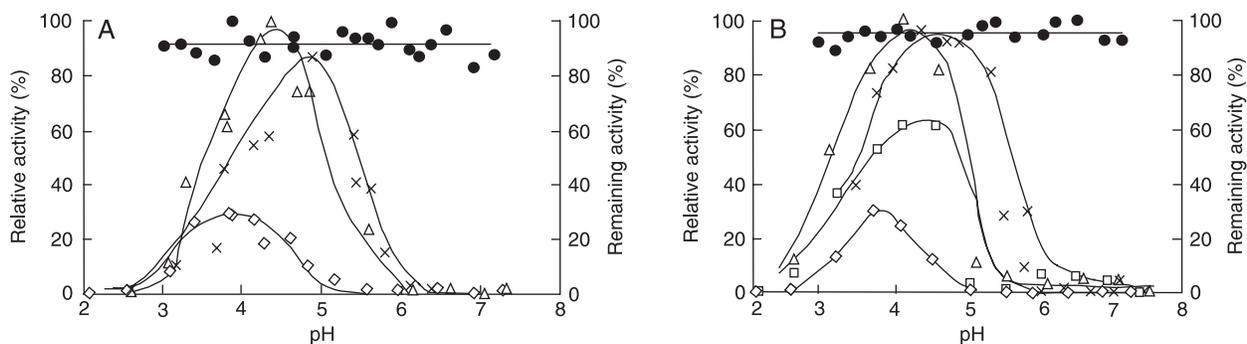


Figure 4. Effect of pH and ionic strength (μ) on activity of Mdl1 (*A*) and Mdl2 (*B*). $\mu = 0.2$ (lozenges), 0.1 (squares), 0.07 (triangles) and 0.02 (symbol X). Each point is the average of four lysozyme activity determinations using a suspension of *Micrococcus lysodeikticus* (1 mg/mL) as substrate. pH stability of Mdl1 and Mdl2 (circles) was checked by incubating enzymes in different pH values for a time (15 min) identical to that used in activity determination. After that, remaining activity was measured. Both enzymes are essentially stable at pH 3.0-7.0.

from 0.07 to 0.25, a condition that resulted in a change of the pH optimum of the activity upon *M. lysodeikticus* (Figure 5). The same was observed for MdL1 when the pH optimum (4.9) at $\mu = 0.07$ (Figure 5) was compared with the one previously reported (4.9) at $\mu = 0.25$ (14).

Steady-state kinetic parameters (V_{\max} and K_m) were also determined at pH optimum and at different ionic strengths using *M. lysodeikticus* as the substrate (Figure 6). MdL2 had higher K_m and V_{\max} than MdL1, regardless of the ionic strength used. In addition, increases of the ionic strength reduced V_{\max} and the affinity of both enzymes for the bacterial cell wall used as substrate.

Discussion

The amino acid sequence deduced from MdL1 cDNA is identical to the lysozyme from *M. domestica* sequenced by Ito et al. (24). Moreover, theoretical pI of the MdL1 (7.7) is similar to that of 7.9 obtained for the major lysozyme from *M. domestica* midgut by isoelectric focusing (13). The comparison between MdL1 and MdL2 sequences (identity of 70%) and pI (7.7 vs 6.7) showed that MdL2 had not been previously reported, although Lemos et al. (13) also de-

scribed a minor low pI (5.5) lysozyme. MdL2 has low pI (6.7) and acidic pH optimum, common traits of digestive lysozymes (11,15), which suggests that MdL2 is also a digestive enzyme.

pH effect on MdL1 and MdL2 activity

MdL1 and MdL2 are stable at pH levels ranging from 3 to 7.3. Within these pH limits, an increase in ionic strength results in a decrease in the lytic activity and in a shift of the pH optimum towards acidic values (Figure 4). These effects, already observed in *M. domestica* (13), *D. melanogaster* (12) and mammalian digestive lysozymes (7), are also found in non-digestive lysozymes from chicken and humans (25-27). However, ionic strength variation shifts the pH optima of non-digestive lysozymes from an alkaline region (about pH 8) to an acidic one (about pH 6), whereas for digestive lysozymes this shift occurs within an acidic pH range, as observed for MdL1 and MdL2 (Figure 4) and other digestive lysozymes: 4.7 to 3.1 for LysD from *D. melanogaster* (12) and 7.0 to 4.9 for lysozyme from *Bos taurus* stomach (7).

The pH optimum shift results from the effect of the ionic strength on the electrostatic potential of the bacterial cell

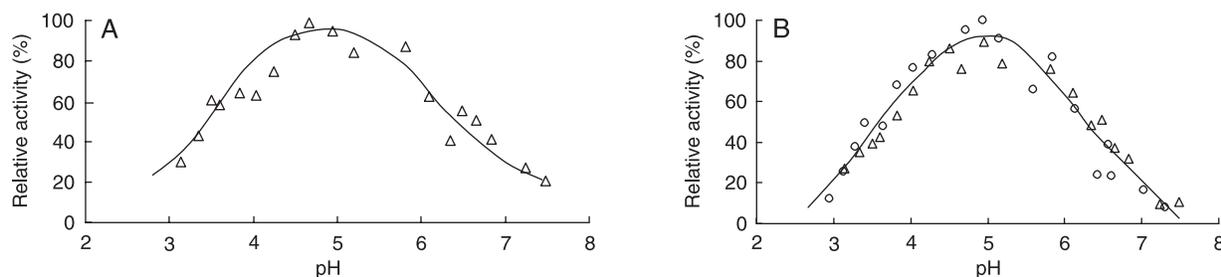


Figure 5. Effect of pH and ionic strength (μ) on activity of MdL1 (A) and MdL2 (B) upon 4-methylumbelliferyl N-acetylchitotrioside (MUNAG3). $\mu = 0.07$ (triangles) and 0.25 (circles).

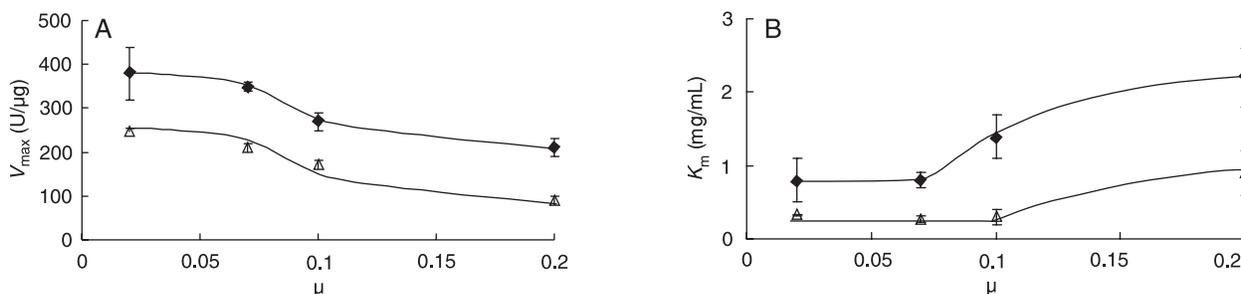


Figure 6. Effect of ionic strength (μ) on kinetic parameters V_{\max} (A) and K_m (B) of MdL1 (triangles) and MdL2 (lozenges). Activity determinations were performed at pH optimum for each μ using a suspension of *Micrococcus lysodeikticus* as substrate. Equal amounts of MdL1 and MdL2 were used in all determinations.

wall (27). The *M. lysodeikticus* cell wall is negatively charged from pH 3 to 12 (28); thus, the H^+ concentration on its surface tends to be higher than in the medium. This pH gradient may be canceled by increasing of ionic strength, because other cations may also interact with the bacterial cell wall and shield its negative charges. Therefore, at low ionic strengths, the pH on the surface of the bacterial cell wall tends to be lower than that of the medium, whereas at high ionic strengths they tend to be similar (27). Since the enzymatic activity of MdL1 and MdL2 is actually affected by the pH on the bacterial surface, their maximum activity at low ionic strength is detected at a higher pH than the one to which they are actually submitted in the bacterial surface. As ionic strength increases, the pH gradient disappears, and the lysozyme pH optima shift towards lower values.

In agreement with that view, the acidic pH optimum (4.9) of MdL1 and MdL2 activity upon a non-charged substrate (MUNAG3) was not affected by ionic strength (Figure 5). This supports the view that the shift of pH optimum due to ionic strength variation is observed only when bacterial cell walls are used as substrates for the lysozymes. Furthermore, the data indicate that the acidic pH optimum of the lytic activity is an intrinsic property of these enzymes. Interestingly, the pH optimum of MdL1 and MdL2 for lytic activity is even more acidic than that of mammalian digestive lysozymes at the same ionic strength (7).

Comparing the amino acid sequences of digestive and non-digestive lysozymes, Regel et al. (12) observed that residue S98 (MdL1 numbering) is conserved in digestive lysozymes. They proposed that the presence of S98 is a determinant cause of the acidic pH optimum of those lysozymes. However, S98 is replaced with a lysine in MdL2, which suggests that S98 residue is probably not associated with the acidic pH optimum of lysozymes. Indeed, in accordance with this result, the comparison of lysozyme sequences revealed that S98 is also present on non-digestive lysozymes from bird eggs, including HEWL (Figure 2), which do not have acidic pH optima.

In HEWL, residues Q57, W108, V109, and A110 form a hydrophobic pocket around catalytic acid/base E35,

whereas N46, D48, S50, and N59 define a hydrophilic environment for catalytic nucleophile D52 (2). Structural data for MdL1 revealed that some of these residues, D48, V109 and A110, are replaced with N46, S106 and T107, respectively, which suggests that changes in the environment of the catalytic residues may play a role in the determination of the acidic pH optimum of MdL1 (14). These replacements are also found in MdL2 (Figure 2), which adds support to the hypothesis that they are important in the determination of the pH optimum in digestive lysozymes.

Substrate affinity of MdL1 and MdL2

Increases in ionic strength result in similar decreases of V_{max} for both MdL1 and MdL2 (Figure 6), which suggest that unproductive ES complexes might be formed at high ionic concentrations. These complexes might result from an ineffective binding between E and S because at high ionic concentrations, the charges of these lysozymes and their substrate were partly screened and the electrostatic interaction between them was consequently weakened. In agreement with that, at pH optimum and high ionic strength, MdL1 and MdL2 have higher K_m values (Figure 6) and are positively charged ($pI = 7.7$ and 6.7 , respectively) and their substrate (*M. lysodeikticus* wall) has a negative charge (28).

Moreover, substrate affinity of MdL2 is lower than that of MdL1 regardless of ionic strength (Figure 6). The analysis of sequence alignment using data of MdL1 structure (14) showed that putative residues forming MdL1 and MdL2 subsites are highly conserved (Figure 2). Therefore, differences in substrate affinity of MdL1 and MdL2 may not be explained by changes of the residues forming their active sites. The pI difference indicates that MdL2 is less positively charged than MdL1, and that the electrostatic interactions between MdL2 and *M. lysodeikticus* walls might be weaker than the interactions with MdL1. Therefore, the electrostatic interactions involving charged residues distributed on the MdL1 and MdL2 overall structure may play a role in the determination of their substrate affinity.

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