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A single histidine residue modulates enzymatic activity in acidic mammalian chitinase

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Abstract **Mammals express two active chitinases, chitotriosidase and AMCase. Only AMCase displays an extremely acidic pH optimum, consistent with its observed presence in the gastrointestinal tract. A structural model of AMCase reveals the presence of a conserved histidine residue in the active site. Mutational analyses and molecular dynamics simulations show that His187 is responsible for the acidic optimum and suggest pH dependent modulation of the reaction mechanism that is unique to AMCases. Concluding, His187 is a crucial structural component of the active site of AMCase and this unique feature may serve as a lead for the development of specific inhibitors.**

Keywords: Chitinase; AMCase; Chitotriosidase; Chitin; Molecular dynamic simulation

1. Introduction

Chitinases are enzymes able to hydrolyze chitin, the linear polymer of β-1,4-linked *N*-acetyl glucosamine (GlcNAc), which is synthesized by a variety of lower organisms for structural purposes [1]. Despite the absence of endogenous chitin, two active chitinases are expressed in mammals. Investigations into the lysosomal storage disorder Gaucher disease serendipitously resulted in the identification of the first mammalian chitinase, chitotriosidase [1–3]. Soon after the second chitinase was discovered which due to its acidic pH optimum was named acidic mammalian chitinase (AMCase) [4]. Both chitinases are 50 kDa proteins, consisting of a 39 kDa catalytic region separated from a chitin-binding domain by a hinge region [4,5]. They both show considerable homology to chitinases from lower organisms and are members of glycoside hydrolase family 18 according to the classification by Henrissat [6]. In man, chitotriosidase is the sole chitinase present in the circulation, consistent with its recently observed anti-fungal activity [7]. Despite this function in the innate immune system, in man a frequent mutation occurs, rendering approximately 6% of most ethnic groups completely deficient in the enzyme [8]. Investigations into the tissue-specific expression of chitinase mRNA and protein have revealed marked inter-species differences between mice and man [9]. In both species AMCase is expressed in the lung and gastro-intestinal tract. The occurrence of acidic chitinases in the stomach of *Xenopus* and *Gallus* species suggests the protein to have evolved as a result of a gene duplication early in tetrapod evolution [10].

A study of the kinetics of the enzyme revealed the protein to be able to catalyze the hydrolysis of chitin substrates as well a the reverse synthesizing reaction due to a phenomenon common to family 18 glycoside hydrolases known as transglycosylation [11,12]. Importantly, AMCase has recently attracted considerable attention due to a report linking the enzyme to pathogenesis of asthma [13]. The protein was reported to be elevated in a mouse model of bronchial asthma. Moreover, inhibition with the transition-state analogue allosamidin ameliorated the Th2 driven, IL13 dependent inflammation, suggesting chitinase activity to play a role in the disease, even in the absence of chitin. Several genetic variants were later proposed to be partly responsible for predisposition to the disease [14]. The precise role of AMCase in immune-mediated diseases is still far from clear since a later report suggested chitinase activity to exert a benificial effect by negatively regulating chitin-induced tissue infiltration of innate immune cells associated with allergy [15].

Crystal structures of several family 18 chitinases have been solved, including that of chitotriosidase. The protein adopts a $(\beta/\alpha)_8$ barrel fold, one of the most versatile folds known. Despite its fairly compact structure, the molecule shows an elongated cleft thought to be capable of binding long chitin-oligomers, compatible with the enzymes presumed endo-chitinolytic activity [16]. Co-crystallization experiments of chitotriosidase with the chitin analogue allosamidin, a micromolar inhibitor, revealed extensive electrostatic interactions and hydrogen bonding as well as hydrophobic stacking interactions between aromatic residues and the hydrophobic acetyl moiety of the chitin-analogue to allow for substrate binding [17].

The reaction mechanism of family 18 glycoside hydrolases was previously shown to proceed via substrate assisted catalysis, based on experiments performed on a bacterial chitinase, Chitinase B [18]. The role of Asp117, located two amino acids from the catalytic Glu119, in substrate binding is thought to be pivotal, in that it changes conformation upon binding of the substrate. pK_a calculations combined with mutational analysis performed on a homologous chitinase later indicated Asp115 and Asp117 to share a proton at all physiologically relevant pH's in the native enzyme, with Asp117 capturing the proton

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Abbreviations: AMCase, acidic mammalian chitinase; MD, molecular dynamics; 4MU, 4-methylumbelliferyl

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upon binding of the substrate, leaving Asp115 deprotonated [19,20]. Subsequently, Glu119 is responsible for protonation of the glycosidic bond, after which hydrolysis takes place.

No structural information has been reported on AMCase to date. However, sequence homology within family 18 chitinases suggests a common reaction mechanism. This study investigates the molecular origin of the acidic optimum of acidic mammalian chitinase by adopting a molecular modelling approach combined with molecular dynamics (MD) simulations, revealing adaptions necessary for functioning in an acidic environment.

2. Materials and methods

2.1. Homology modelling and molecular dynamics

The models of human and murine AMCase were based on the crystal structure of native chitotriosidase 1LQ0 (resolution 2.20 Å). Initial modelling of the AMCase structures was accomplished online using the SWISSPROT server [21]. In order to construct a model of the H187N mutant, the histidine at position 187 was converted into a asparagine using the program Deepview [21]. Both the native and modified models were subjected to energy minimalization in GROMACS version 3.3.1 with the GROMOS96 forcefield [22] using the steepest decent method. The quality of the models based on a variety of stereochemical parameters was determined by PROCHECK [23]. Surface electrostatic potentials of the modelled structures were calculated and visualized using Deepview [21]. MD was performed as described in the supplemental information.

2.2. Mutagenesis, recombinant protein expression and enzymatic assays

The H187N point mutation was introduced directly into the wild-type murine AMCase cDNA in the expression plasmid, pcDNA3(1), using the high fidelity Quik-Change™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. Large-scale production and purification of the wild-type and mutant cDNA expression plasmids were performed using Promega Wizard Plus Midiprep kits. Recombinant expression in COS-7 cells was accomplished as described earlier [3]. For the enzyme activity assay

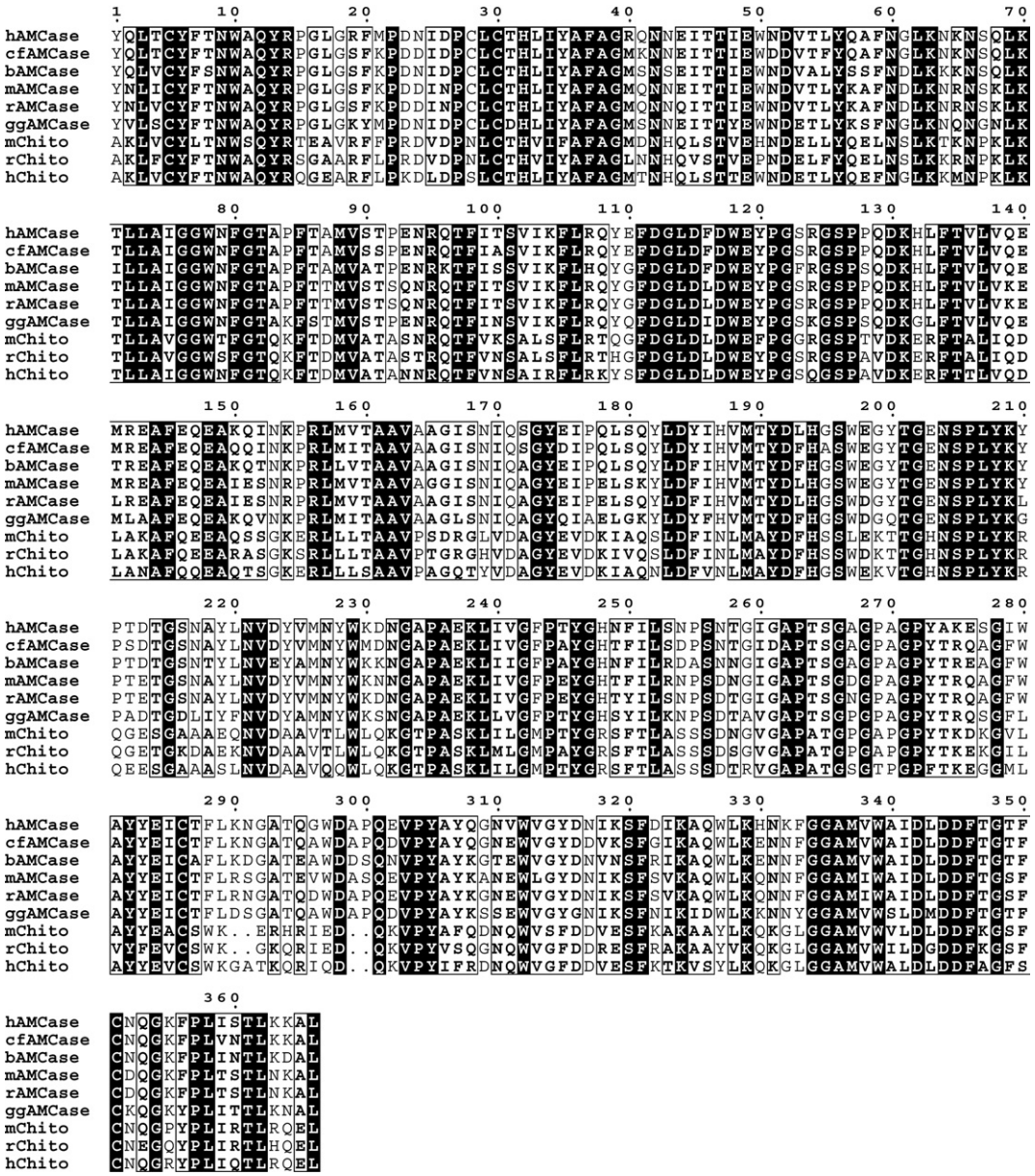

Fig. 1. Alignment of the 39 kDa domain of vertebrate chitotriosidase and AMCase. h – *Homo sapiens*; cf – *Canis familiaris*; b – *Bos taurus*; m – *Mus musculus*; r – *Rattus rattus*; gg – *Gallus gallus*. Boxing is according to RISLER similarity scoring.

with 4-MU-substrates, 25 μl medium and 100 μl substrate mixtures were incubated for 1 h at 37 °C. The substrate mixtures contained 0.111 mM 4MU-deoxychitobiose and 1 mg/ml BSA in McIlvain buffer, at different pH. Reactions were stopped with 2.0 ml of 0.3 M glycine NaOH buffer pH 10.6 and the formed 4MU (4-methylumbelliferyl) was detected fluorometrically (excitation at 445 nm; emission at 366 nm). Only less than 10% difference in the duplicates was allowed. One unit (U) of activity is defined as 1 nmol of substrate hydrolyzed per hour.

3. Results and discussion

3.1. Homology comparisons and overall AMCase structure

In order to gain insight into structure–function relationships we aligned both chitinases of various species. The alignment shown in Fig. 1 shows substantial overall conservation, in particular of the active site sequence, yet orthologue specific amino acids exist at various positions. In order to assess their importance in the catalytic mechanism we created models for AMCase based on the published chitotriosidase structure [16]. The overall structure shows high similarity with the experimentally determined crystal structure of chitotriosidase. This is reflected in a low root mean square deviation (RMSD) for Cα atoms between the mouse AMCase model and chitotriosidase structure of 0.54 Å. PROCHECK detected no major deviations from optimal geometry.

3.2. Amino acid differences between AMCase and chitotriosidase

Amino acid substitutions resulting in increased protein stability or increased enzymatic activity at low pH should be distinguished. The majority of substitutions in AMCases compared to chitotriosidase reside on the protein surface. This suggests they are adaptions conferring stability rather than adaptions directly modulating the reaction mechanism. Indeed, calculation of the surface potential of both mouse and human AMCase shows that its substitutions result in a predominantly negative charge at the surface, as compared to chitotriosidase (Fig. 2). This most likely restricts intramolecular repulsion as a consequence of excessive protonation at low pH. Evolution of digestive lysozymes has followed a similar pattern [24].

Additionally, since AMCase contains two additional cysteines compared to chitotriosidase, a third disulfide bond most likely enhances the fold integrity in an acidic environment. Indirect evidence for this is provided by the observation that AMCase shows a different electrophoretic behaviour in the absence of a reducing agent, suggesting a difference in disulfide bonding between the two mammalian chitinases [4].

3.3. Influence of histidine 187

The mapping of sequence differences onto the modelled structures revealed that only a single paralogue specific substitution, His187, is located close to the active site. This histidine is conserved in all AMCases (Fig. 1). Replacing His187 with an asparagine in murine AMCase, the amino acid conserved at this position in chitotriosidase, revealed a clear difference in pH-dependent activity compared to wild-type enzyme. Strikingly, activity at low pH is nearly abolished, wheras the near-neutral activity is affected far less (Fig. 3).

In order to elucidate the role of this residue, MD simulations were performed on several systems: AMCase (both human and murine) with fully protonated His187, AMCase with deprotonated His187, the mutant murine AMCase His187Asn and chitotriosidase. All systems show considerable rigidity of secondary structure during the 10 ns runs, as shown by r.m.s. fluctuation (RMSF, a measure for flexibility) of 0.05–0.25 nm, consistent with the compact, highly stabilized structure of the $(\beta/\alpha)_8$ barrel. As expected, in view of hydrolase activity, the catalytic glutamic acid (Glu119), is accessible to solvent.

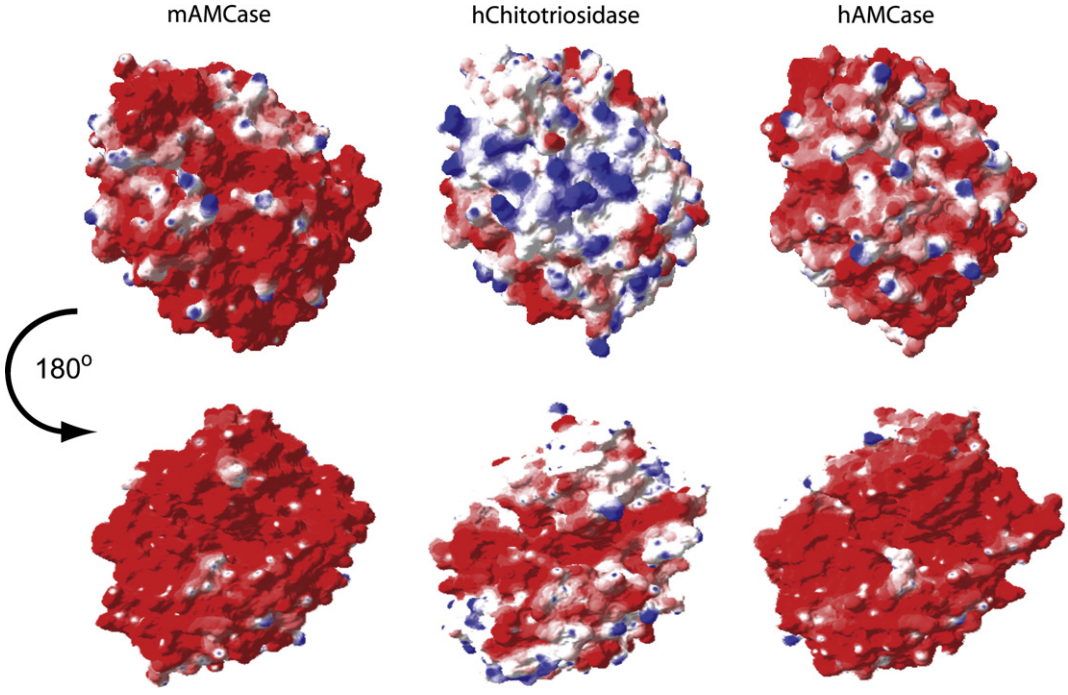

Fig. 2. Surface potential of murine AMCase, human chitotriosidase and human AMCase. Red and blue represent a negative and positive surface charge, respectively (±2.5 kT/e).

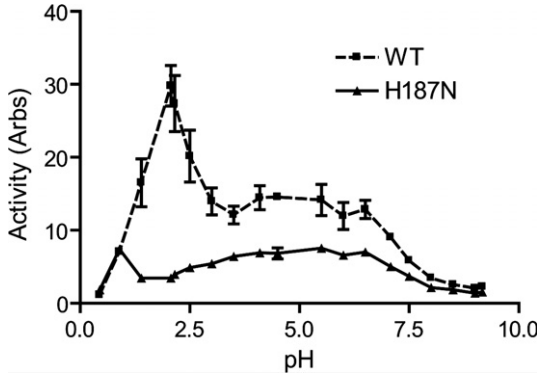

Fig. 3. pH activity profile of wild-type and mutagenized His187Asn murine AMCase. Activity was measured with 4MU-deoxychitobioside as substrate as described in Section 2.

Importantly, the simulations show a clear salt bridge interaction between deprotonated Asp117 and protonated His187 that remains constant during the full 10 ns. This distance is increased and fluctuates in both the mutant and chitotriosidase, suggesting that this strong interaction is absent in these enzymes (Fig. 4). The results were highly similar simulating both murine and human AMCase, suggesting the role of His187 to be ubiquitous in both enzymes.

3.4. Acid pH optimum

A mechanistic explanation for the acid optimum and role of His187 can be given based on the reaction mechanism postulated earlier [20]. One of the pivotal steps in catalysis is the rotation of Asp117, preceding binding of the substrate by allowing formation of an H-bond with the *N*-acetyl moiety of the chitinous substrate. This can only be achieved by disruption of the stabilized system in which Asp115 and Asp117 share a proton. Such a role can easily be envisioned for protonated His187 based on the fact that it is located close to the Asp115 carboxylate (Fig. 5).

For the observed pH optimum of mouse AMCase to be explained by protonation of His187, the effective pK_a should be dramatically decreased, however this is not without precedent. pK_a values of histidines are known to vary greatly depending on their electrostatic environment [25]. In the case

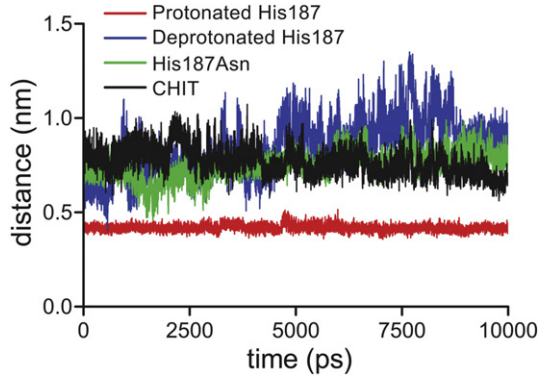

Fig. 4. Distances in time between Asp117 and His187/Asn187. Distances were calculated for (4) systems: (1) murine AMCase with protonated His187; (2) murine AMCase with deprotonated His187; (3) His187Asn mutant murine AMCase; (4) chitotriosidase. The distances plotted are between the Cγ atoms of both amino acids. The values shown are representative for each of three independent MD runs.

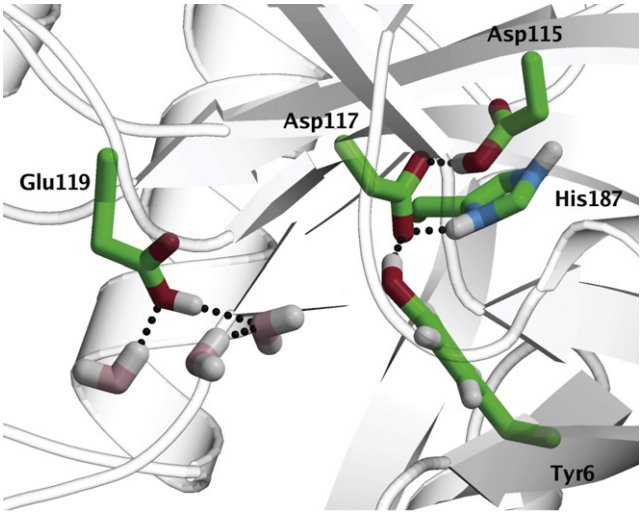

Fig. 5. Location of His187 within the active site of mouse AMCase. MD snapshot showing interactions between the buried (protonated) His187, (deprotonated) Asp117, Asp115 and Tyr6. The solvated catalytic Glu119 is also shown.

of *Bacillus circulans* xylanase, for instance, the pK_a value of a histidine residue (His149) is estimated to be <2.3, based on NMR titration experiments [26]. This is likely in part due to the hydrophobic environment surrounding the residue. More importantly perhaps, the histidine is inaccesible to solvent. Similarly, in AMCase, the presence of several "acidic" hydroxyl (Tyr6) and carboxylic acid groups (Asp115 and Asp117) in close vicinity may result in a change in effective pK_a of the histidine (Fig. 5). Furthermore, visual inspection of the AMCase simulations shows that His187 is inaccessible to bulk water in both protonation states. The lack of stabilizing water molecules close to His187 is likely to favor a neutral state at low pH. Although our study renders proof for an important role of His187 in the extremely acidic pH optimum of mouse AMCase, other structural features most likely also have an impact. For example, the acidic activity of human AMCase is not as pronounced as that of mouse AMCase, despite the presence of His187 [4,12]. As the surface potential of mouse AMCase is significantly lower (Fig. 2), the loss of structural stability at very low pH may limit the extreme acidic activity of human AMCase. Thus concluding His187 represents a major difference in the active site of AMCase compared to that of chitotriosidase. The amino acid allows activity at extremely low pH, provided that the overall protein fold is stable at this extreme condition. The unique His187 feature of AMCases may serve as a lead for the development of specific inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.032.

References

[1] Bussink, A.P., van Eijk, M., Renkema, G.H., Aerts, J.M. and Boot, R.G. (2006) The biology of the Gaucher cell: the cradle of human chitinases. Int. Rev. Cytol. 252, 71–128.

[2] Hollak, C.E., van Weely, S., van Oers, M.H. and Aerts, J.M. (1994) Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. J. Clin. Invest. 93, 1288–1292.

[3] Renkema, G.H., Boot, R.G., Muijsers, A.O., Donker-Koopman, W.E. and Aerts, J.M. (1995) Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. J. Biol. Chem. 270, 2198–2202.

[4] Boot, R.G., Blommaart, E.F., Swart, E., Ghauharali-van der Vlugt, K., Bijl, N., Moe, C., Place, A. and Aerts, J.M. (2001) Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J. Biol. Chem. 276, 6770–6778.

[5] Boot, R.G., Renkema, G.H., Strijland, A., van Zonneveld, A.J. and Aerts, J.M. (1995) Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. J. Biol. Chem. 270, 26252–26256.

[6] Henrissat, B. (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280, 309–316.

[7] van Eijk, M., van Roomen, C.P., Renkema, G.H., Bussink, A.P., Andrews, L., Blommaart, E.F., Sugar, A., Verhoeven, A.J., Boot, R.G. and Aerts, J.M. (2005) Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int. Immunol. 17, 1505–1512.

[8] Boot, R.G., Renkema, G.H., Verhoek, M., Strijland, A., Bliek, J., de Meulemeester, T.M., Mannens, M.M. and Aerts, J.M. (1998) The human chitotriosidase gene. Nature of inherited enzyme deficiency. J. Biol. Chem. 273, 25680–25685.

[9] Boot, R.G., Bussink, A.P., Verhoek, M., de Boer, P.A., Moorman, A.F. and Aerts, J.M. (2005) Marked differences in tissue-specific expression of chitinases in mouse and man. J. Histochem. Cytochem. 53, 1283–1292.

[10] Bussink, A.P., Speijer, D., Aerts, J.M. and Boot, R.G. (2007) Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. Genetics 177, 959–970.

[11] Aguilera, B., Ghauharali-van der Vlugt, K., Helmond, M.T., Out, J.M., Donker-Koopman, W.E., Groener, J.E., Boot, R.G., Renkema, G.H., van der Marel, G.A., van Boom, J.H., Overkleeft, H.S. and Aerts, J.M. (2003) Transglycosidase activity of chitotriosidase: improved enzymatic assay for the human macrophage chitinase. J. Biol. Chem. 278, 40911–40916.

[12] Chou, Y.T., Yao, S., Czerwinski, R., Fleming, M., Krykbaev, R., Xuan, D., Zhou, H., Brooks, J., Fitz, L., Strand, J., Presman, E., Lin, L., Aulabaugh, A. and Huang, X. (2006) Kinetic characterization of recombinant human acidic mammalian chitinase. Biochemistry 45, 4444–4454.

[13] Zhu, Z., Zheng, T., Homer, R.J., Kim, Y.K., Chen, N.Y., Cohn, L., Hamid, Q. and Elias, J.A. (2004) Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 304, 1678–1682.

[14] Bierbaum, S., Nickel, R., Koch, A., Lau, S., Deichmann, K.A., Wahn, U., Superti-Furga, A. and Heinzmann, A. (2005) Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. Am. J. Respir. Crit. Care. Med. 172, 1505–1509.

[15] Reese, T.A., Liang, H.E., Tager, A.M., Luster, A.D., van Rooijen, N., Voehringer, D. and Locksley, R.M. (2007) Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature 447, 92–96.

[16] Fusetti, F., von Moeller, H., Houston, D., Rozeboom, H.J., Dijkstra, B.W., Boot, R.G., Aerts, J.M. and van Aalten, D.M. (2002) Structure of human chitotriosidase. Implications for specific inhibitor design and function of mammalian chitinase-like lectins. J. Biol. Chem. 277, 25537–25544.

[17] Rao, F.V., Houston, D.R., Boot, R.G., Aerts, J.M., Sakuda, S. and van Aalten, D.M. (2003) Crystal structures of allosamidin derivatives in complex with human macrophage chitinase. J. Biol. Chem. 278, 20110–20116.

[18] Terwisscha van Scheltinga, A.C., Armand, S., Kalk, K.H., Isogai, A., Henrissat, B. and Dijkstra, B.W. (1995) Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis. Biochemistry 34, 15619–15623.

[19] Synstad, B., Gaseidnes, S., van Aalten, D.M., Vriend, G., Nielsen, J.E. and Eijsink, V.G. (2004) Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. Eur. J. Biochem. 271, 253–262.

[20] van Aalten, D.M., Komander, D., Synstad, B., Gaseidnes, S., Peter, M.G. and Eijsink, V.G. (2001) Structural insights into the catalytic mechanism of a family 18 exo-chitinase. Proc. Natl. Acad. Sci. USA 98, 8979–8984.

[21] Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.

[22] Lindahl, E., Hess, B. and van der Spoel, D. (2001) GROMACS 3.0: a package for molecular simulation and trajectory analysis. J. Mol. Model. 7, 306–317.

[23] Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Cryst. 26, 283–291.

[24] Regel, R., Matioli, S.R. and Terra, W.R. (1998) Molecular adaptation of Drosophila melanogaster lysozymes to a digestive function. Insect Biochem. Mol. Biol. 28, 309–319.

[25] Edgcomb, S.P. and Murphy, K.P. (2002) Variability in the p*K*a of histidine side-chains correlates with burial within proteins. Proteins 49, 1–6.

[26] Plesniak, L.A., Connelly, G.P., Wakarchuk, W.W. and McIntosh, L.P. (1996) Characterization of a buried neutral histidine residue in Bacillus circulans xylanase: NMR assignments, pH titration, and hydrogen exchange. Protein Sci. 5, 2319–2328.