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Purification and Characterization of Acidic Chitinases from Gizzards of Broiler (*Gallus gallus L.*)

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Acidic chitinases from the gizzards of a broiler were purified to homogeneity, using precipitation with $(NH_4)_2SO_4$, ion exchanger chromatography, gel filtration, chromatofocusing and hydrophobic interaction chromatography. The enzymes, GAC1 and GAC2, were purified 180- and 194-folds with a recovery of 4.9% and 2.7%, respectively. The molecular mass of GAC1 and GAC2 were 48.2 kDa and 57.8 kDa, respectively. Chromatofocusing resulted in a p*I* of 3.1 for both enzymes. The purified enzymes were endochitinases that were devoid of β-*N*-acetylglucosaminidase and lysozyme activity. Kinetic studies using [^{3}H]chitin indicate that GAC1 has a K_m and V_{max} of 1.97 mg/ml and 185 mg/mg protein/h, respectively. The GAC2 has a K_m and V_{max} of 0.42 mg/ml and 92.3 mg/mg protein/h, respectively at optimal pH and temperature (pH 5.0 and 60°C). When the pentamer and hexamer of *N*-acetylglucosamine (GlcNAc) were used as a substrate, the major product by GAC1 was the dimer of GlcNAc with a differential accumulation of the monomer and trimer, depending upon the substrate. However, the GAC2 produced the dimer and trimer in an equal quantity, regardless of the substrate used. The first 9 NH_2-terminal amino acid residues of the purified gizzard chitinase GAC1 and GAC2 shared a 100% homology. The first 25 NH_2-terminal amino acid residues of GAC1 also shared 55-60% homology with animal chitinases and some animal proteins, such as whey protein and oviduct-specific proteins. However, little homology was found with either microbial and plant chitinases, or egg white lysozyme.

Keywords: Broiler, Chitinase, Gizzards, NH_2-terminal, Purification.

Introduction

Chitin, chitosan and their low molecular weight oligosaccharides have recently received much attention due to a broad variety of industrial and biomedical applications. The enzymes responsible for the hydrolysis of chitin consist of two hydrolysases. They are endo-chitinase (EC 3.2.1.14) and β-*N*-acetylglucosaminidase (EC 3.2.1.30). The chitinases from bacteria, moulds, insects and plants have been purified and relatively well characterized (Flach *et al.*, 1992). This is mainly due to their vital role in these forms of life: (1) energy extraction from the environment, (2) modification of the chitin components in fungi and arthropods (Kramer and Muthukrishnan, 1992), and (3) the stress response system in the plant (Collinge *et al.*, 1993).

In contrast, the chitinases from the gastrointestinal tracts of vertebrates have not been studied sufficiently, except those from fish (Kono *et al.*, 1987). However, studies of the digestive tracts of birds have showed that the sparrow (*Passer domesticus*), the Japanese nightingale (*Liothrix lutea*), the adult chicken (*Gallus gallus*) and the barn owl (*Tyto alba*) have a high chitinolytic activity. However, the wood pigeon (*Columba palumbus*) and the African grey parrots (*Psittacus erithacus*) do not show any chitinolytic activity (Jeuniaux and Cornelius, 1978). Furthermore, these studies demonstrated that *Gallus gallus* and *Liothrix lutea* digested the ingested chitin at the level of 20-50%. Despite its important role in poultry nutrition, bird chitinases have not been extensively investigated.

Following the report of a crude enzyme prepared from the chicken (Han *et al.*, 1997b), we recently investigated the chitinolytic activity of the gizzard and the chyme from *Gallus gallus* for industrial applications (Han *et al.*, 1997a). The crude preparations from the gizzards of the *Gallus gallus* showed a high endochitinolytic activity with good thermostability. To the best of our knowledge, no investigations into the digestive tracts of bird chitinases have been published. In this paper, we describe the purification of

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acidic endochitinases from the gizzard of a broiler, their enzymatic properties and the NH_2-terminal amino acid sequences.

Materials and Methods

Materials Fresh gizzards of the broilers were obtained from a local poultry-processing factory. They were stored at -70°C until used. After washing the gizzard with an extraction buffer containing 0.02 M of citric acid/0.02 M Na_2HPO_4, pH 5.2, the mucous lining was separated from the stomach. All of the packing materials of the chromatography and prepacked columns were purchased from Amersham-Pharmacia (Sweden).

Extraction of the crude enzymes The remaining tissue was cut and homogenized with Ace homogenizer (Nihonseiki, Japan) in the presence of the extraction buffer (1 : 4, w/v). The homogenate was successively centrifuged at 3,000 g for 10 min and at 20,000 g for 20 min in order to obtain the crude enzyme. The crude preparation was precipitated with a 80% saturation with $(NH_4)_2SO_4$. After centrifugation at 20,000 g for 20 min, the precipitate was recovered and dialyzed against the extraction buffer. The protein content was determined by the method of Lowry *et al.* (1951).

Purification procedures The crude preparation was adjusted to pH 4.5 and loaded onto a cation exchanger column (Sources-S, 2.6 cm × 15 cm). The column was extensively washed before elution with a linear gradient of NaCl from 0.0 M to 1.0 M. The unbound fractions that contained the enzyme activity were pooled and concentrated with a UF membrane (cut-off: 10,000) before freeze-drying. After an overnight dialysis against 0.02 M Tris-HCl, pH 7.5, 40 ml of the sample (3 mg/ml) were applied on an anion exchanger column (Source-Q, 2.6 cm × 15 cm). The column was amply washed with the 0.02 M Tris-HCl and eluted with a step-wise gradient of NaCl (0.18 M and 0.38 M). The active fraction was freeze-dried. The protein was dissolved in the 0.02 M Tris-HCl (3 mg/ml) and 500 µl were applied on a gel filtration column (Superdex G200, 1.6 cm × 65 cm). Elution was performed at a rate of 0.5 ml/min. The fraction containing chitinase activity was pooled and freeze-dried. The powder was dissolved in a 0.025 M bisTris-HCl, pH 6.3, and loaded on a chromatofocusing column (Mono-P HR, 0.5 cm × 20 cm), which was pre-equilibrated with 0.025 M bisTris-HCl, pH 6.3. A polybuffer 74 was diluted 10 times and adjusted to pH 3.2 and 2.0, respectively. The column was eluted successively with the diluted Polybuffers at pH 3.2 and 2.0. Solid $(NH_4)_2SO_4$ was added to the active fractions at a concentration of 1.0 M. The resulting solution was applied directly to a hydrophobic interaction column (Phenyl-Sepharose HP, 1 ml) that was pre-equilibrated with 1.0 M $(NH_4)_2SO_4$ in 0.02 M Tris-HCl, pH 7.5. The column was eluted with a linear gradient of $(NH_4)_2SO_4$ from 1.0 M to 0.0 M. The eluted chitinases were pooled and kept at 4°C.

Electrophoresis and detection of chitinases and proteins on the gel The 12.5% SDS-PAGE was performed in the presence of a 0.01% glycol chitin, according to Laemmli (1970). The enzyme activity was detected with a Calcofluor white M2R after treatment of the gel with Triton X-100, as described by Trudel and Asselin (1989). Proteins were visualized with 0.1% Coomassie brilliant blue R250, or silver nitrate staining according to the method of Wray *et al.* (1981).

Enzyme assays The chitinase activity was measured using [^{3}H]chitin that was prepared according to the method reported by Molano *et al.* (1977). [^{3}H]chitin was suspended in the extraction buffer (1 mg/ml, w/v), and 100 µl were taken under continuous agitation. The 588 dpm were equivalent to 1 µg of dry chitin. The radioactivity was quantified with a β-scintillation counter (Tricarb 1600, Packard). For the kinetic study, 18,000 dpm to 1,500,000 dpm of [^{3}H]chitin was added to 100 µl of the purified enzyme solution (4.0 µg/ml of 0.05 M McIlvane buffer, pH 5.0). The final volume was adjusted to 1.0 ml. After a 2 h incubation at 60°C at pH 5.0, boiling the solution at 100°C for 10 min stopped the reaction. Since [^{3}H]chitin was used as substrate, K_m and V_{max} were expressed mg/ml and mg/mg protein/h instead of mM. When oligomers of *N*-acetylglucosamine (GlcNAc) were used as substrate, 1 mg of oligomers was added to 1 ml of the McIlvane buffer (0.05 M, pH 5.0) and aliquots of 100 µl were removed at appropriate time intervals. One unit of enzyme activity is defined as the amount of enzyme that is required in order to produce 1 µmol of monomers per minute at 60°C. The β-*N*-acetylglucosaminidase assays were carried out by adding 100 µl of the purified enzyme solution to 0.2 ml of 4 mM ρ-nitrophenyl-β-*N*-acetylglucosaminide in a McIlvane buffer (0.05 M, pH 5.0). The final volume of incubation was adjusted to 0.5 ml. After 1 h incubation at 37°C with shaking, 2 ml of the 0.2 M sodium carbonate was added to the reaction mixture and the liberated ρ-nitrophenol were determined by measuring absorbance at 420 nm.

The lysozyme activity was measured according to the method described by Martin (1991). To 1 mg of the cell wall from *Micrococcus lysodeikticus*, 0.4 µg of the purified enzyme were added. After 2 h incubation at 37°C, the absorbency was measured at 570 nm.

Analysis of reaction products by TLC The reaction mixture was applied on a pre-coated silica gel G255 plate and developed twice with a solvent system of isoamyl-alcohol : ethanol : water : NH_4OH (50 : 60 : 36 : 1, v/v). After spraying a mixture of 1% *o*-aminophenol : H_3PO_4 : water (1 : 2 : 1, v/v), the plate was heated to 110°C for 1 h in order to obtain color development products. Densitometry was done with a Phospho-Image analyzer system GS-505 (BioRad, USA).

Determination of NH_2-terminal amino acid sequence The purified enzyme was blotted on a PVDF membrane. An automated Edman degradation was performed with a protein/peptide sequencer 471A (Applied Biosystem, USA) according to the method described by the manufacturer.

Results and Discussion

Purification of acidic chitinases, GAC1 and GAC2 Since pH affects the removal of proteins in ion exchange

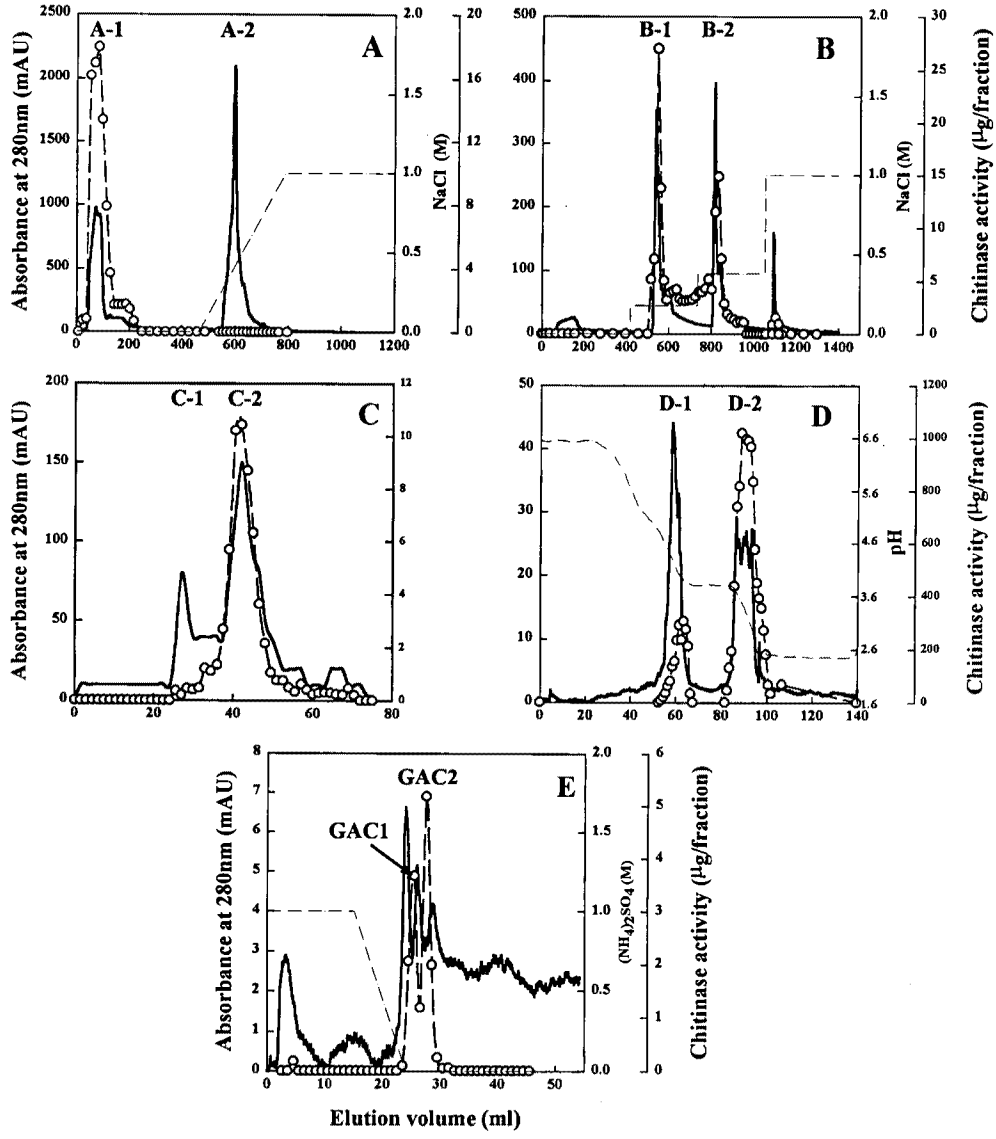

Fig. 1. Liquid chromatography of *Gallus gallus* chitinases. [^{3}H] chitin was used as substrate and 588 dpm correspond to 1 μg of chitin. Fraction volume was 15 ml. (A) Source-S chromatogram of 20,000 g supernatant, (B) Source-Q chromatogram of fraction A-1, (C) Superdex G-200 gel filteration of fraction B-2, (D) Mono-P chromatofocusing of C-2 with Polybuffer 74, (E) HIC of D-2 with Phenyl-Sepharose ——: absorbance at 280 nm, —O—O—: chitinase activity, — - —: Salt/pH gradient

chromatography, pH of the supernatant was adjusted to 6.5, 5.5 and 4.5 before application on Source-S (2.6 cm × 15 cm). The removal of proteins and recovery of chitinases were highest at pH 4.5. More than 95% of the total chitinase activity was found in the unbound A-1 fraction (Fig. 1A). When the unbound A-1 was chromatographied on the Source-Q column, it was separated into two major peaks (B-1 and B-2) that contained chitinase activity (Fig. 1B). Although B-1 contained 43% of the total activity and had a higher specific activity and yield (0.63 mg/mg protein/h, 43%) than B-2 (0.35 mg/ mg protein/h, 16%), B-2 was further purified because the protein bands of this fraction were much simpler than those of B-1 (data not shown).

Gel filtration of the B-2 fraction from Source-Q column resulted in two peaks. C-2 contained most of its chitinase activity (Fig. 1C). No protein was eluted at pH 6.3 at chromatofocusing of C-2 with a Mono-P column (Fig. 1D). The first fraction D-1 with enzyme activity was eluted at 4.1, but showed lower. After stabilizing the column at pH 3.8, a second linear gradient of pH from 3.8 to 2.6 was carried out. Immediately after the gradient formation, D-2 was eluted and contained 80% of total activity of chitinases (Fig. 1D).

The D-2 from chromatofocusing chromatography was subjected to HIC. The GAC1 and GAC2 were obtained (Fig. 1E) and both peaks showed a single band upon SDS-PAGE (Fig. 2A). The GAC1 and GAC2 had a molecular weight of 48.2 kDa and 57.8 kDa, respectively (Fig. 2B). Animal chitinase molecular weights ranged from 35 kDa to 88 kDa

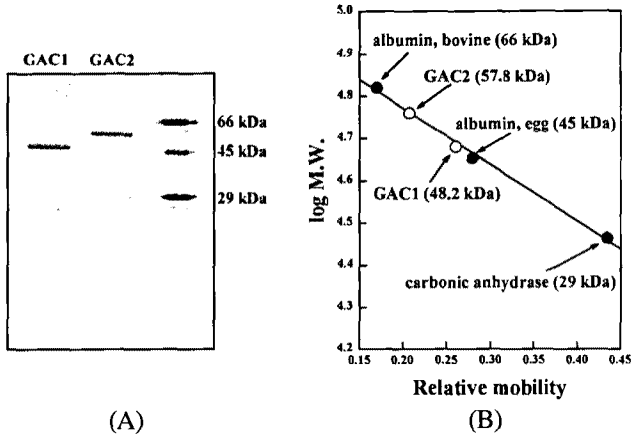

Fig. 2. SDS-PAGE of GAC1 and GAC2, and estimation of their molecular weight. (A) SDS-PAGE of GAC1 and GAC2, (B) Estimation of the molecular weight

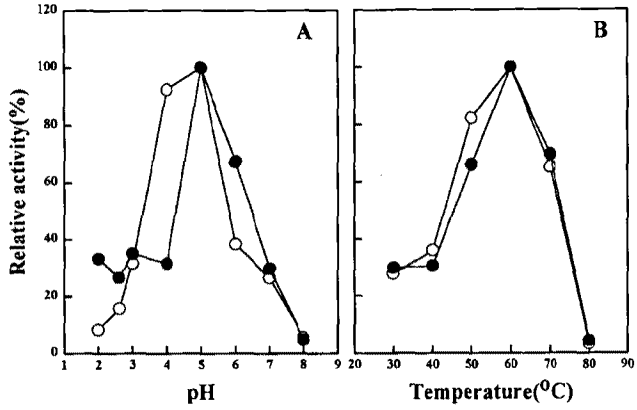

Fig. 3. Effect of pH and temperature on chitinase activity of GAC1 (○) and GAC2 (●). (A) pH effect, (B) Temperature effect

depending upon their origin. A chitinase of 50 kDa was isolated from the stomach of a Japanese eel (*Anguilla japonica*) by Kono *et al.* (1990). Koga *et al.* (1997) purified two chitinases (65 kDa and 88 kDa) from the larvae of a *Bomyx mori*. Lundblad *et al.* (1979) purified a chitinase from bovine serum whose molecular weight is 47 kDa.

The results of the purification are summarized in Table 1. At the final step the 48 kDa and 58 kDa proteins were purified 180- and 197-fold with a recovery of 4.9% and 2.7%, respectively.

Enzymatic properties of the acidic chitinases, GAC1 and GAC2 The optimal pH of GAC1 ranged from 4.0 to 5.0, while that of GAC2 was 5.0 (Fig. 3A). As seen in Fig. 3B, the purified chitinases have a maximal activity at 60°C and lost most of its activity at 80°C. Since a crude enzyme preparation from the gizzard maintained 40% of its activity at 80°C (Han *et al.*, 1997a), some thermostabilizing factors must be removed during purification. The p*I* of GAC1 and GAC2 was calculated to be about 3.1 from chromatofocusing.

The β-*N*-acetylglucosaminidase, or lysozyme activity, was not observed even after prolonged incubations. The lack of β-*N*-acetylglucosaminidase and lysozyme activity is in agreement with the reported insect chitinases (Koga *et al.*, 1983). The K_m and V_{max} of GAC1 were 1.97 mg/ml and 185 mg/mg protein/h, respectively. The GAC2 had a K_m and V_{max} for [^{3}H]chitin of 0.42 mg/ml and 92.3 mg/mg protein/h, respectively (Fig. 4).

When ρ-nitrophenyl-β-*N*-acetylglucosaminide and trimer of GlcNAc were incubated with GAC1 and GAC2 under prolonged incubations, no hydrolysis products were detected under our experimental conditions. After 1 h incubation the formation of monomer, dimer and trimer from the GlcNAc pentamer was 29%, 54% and 17%, respectively. Thereafter the dimer increased steadily to about 80%, while the monomer remained unchanged (30%). In contrast, the formation of the trimer decreased slowly after 1 hour incubation and reached a non-detectable level within 5 hours (Fig. 5A-1). Furthermore, as seen in Fig. 5A-2, the major hydrolysis product of the hexamer with GAC1 was also the dimer (70%). In contrast, the trimer of GlcNAc accounted for 30% of the product, with a trace amount of the GlcNAc monomer. Considering that the GlcNAc trimer was not hydrolyzed by either GAC1 or GAC2, the accumulation of monomer of GlcNAc for the GlcNAc pentamer is quite unexpected (Fig. 5A-1). The GAC2 hydrolyzed the GlcNAc pentamer and hexamer differently. The GAC2 produced the

Table 1. Purification of the acidic chitinases, GAC1 and GAC2

Step		Protein (mg)	Activity (U)	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
Crude extract		3,006	11.52	3.8	100	1
$(NH_4)_2SO_4$ precipitate		1,058	9.3	8.8	81.1	2.3
Source-S		352	8.4	24.0	73.3	6.7
Source-Q		63.3	1.84	29.0	15.9	7.6
Superdex G200		22.1	1.46	66.0	12.7	17.2
Mono-P		6.2	1.16	187.0	10.0	48.7
Phenyl-Sepharose	GAC1	0.82	0.57	688.0	4.9	179.6
	GAC2	0.41	0.31	756.3	2.7	197.4

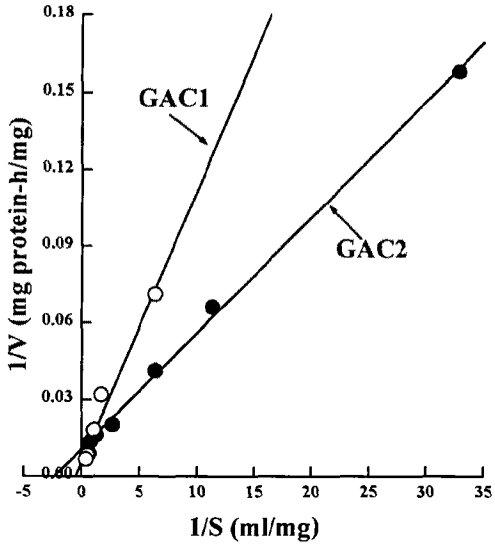

Fig. 4. Lineweaver-Burk plot of GAC1 (○) and GAC2 (●). [^{3}H]-chitin was used as substrate and 588 dpm correspond to 1 μg of chitin.

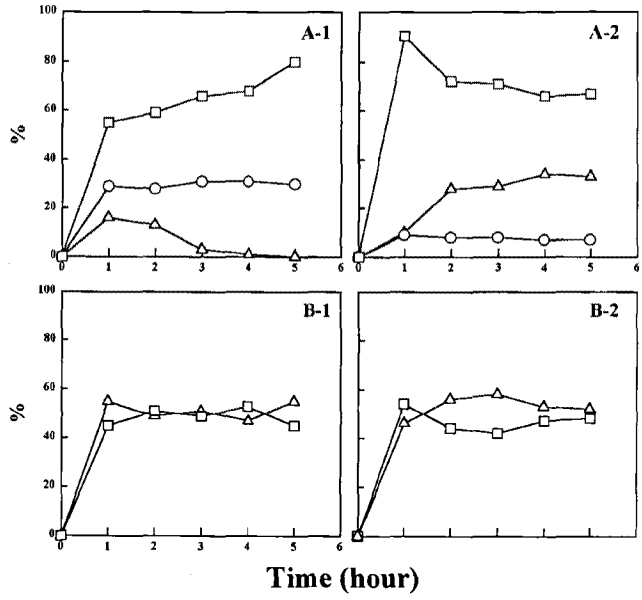

Fig. 5. Formation of reaction products by GAC1 and GAC2. The total of three reaction products (monomer, dimer and trimer) was taken as 100 %. (A) Reaction products of GAC1. A-1: Pentamer of GlcNAc as substrate, A-2: Hexamer of GlcNAc as substrate. (B): Reaction products of GAC2. B-1: Pentamer of GlcNAc as substrate, B-2: Hexamer of GlcNAc as substrate. ○: GlcNAc, ❑: dimer of GlcNAc, △: trimer of GlcNAc.

dimer and trimer in an equal quantity without any detectable formation of GlcNAc, regardless of the substrate used (Fig. 5B-1, B-2). The GAC2 is similar to the *B. mori* chitinases in cleavage patterns (Koga *et al.*, 1997). In contrast the cleavage patterns of GAC1 are different. GAC1 needs to be investigated further.

NH_2-terminal amino acid sequence When the 9 sequences of the NH_2-terminal of GAC1 and GAC2 were compared, both chitinases shared 100% homology (Fig. 6). Compared with the known NH_2-terminal sequence of chitinases from bacteria and plants (Flach *et al.*, 1992), the GAC1 chitinase shared little homology. As shown in Fig. 6, however, GAC1 shared 64%, 63%, 59% and 54% homology with animal chitinases from the *Brugia malayi* (SwissProt P29030), silk worm (*Bombyx mori*) (Koga *et al.*, 1997), a chitotriosidase from human (Renkema *et al.*, 1995) and *Caenorhabditis elegans* (SwissProt Q11174), respectively. Furthermore the purified chitinase also shared 60%, 55-56% homology with bovine cartilage glycoprotein-39 (SwissProt P30922), and oviduct-specific glycoproteins of several species (SwissProt Q62010, P36718, Q28042, Q12889), respectively. The fact

Protein		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	%
Broiler HF1		Y	V	L	S	X	Y	F	T	N	W	A	Q	Y	R	P	G	V	G	S	F	M	P	D	N		100
Brugia malay chitinase		Y	V	R	G	C	Y	Y	T	N	W	A	Q	Y	R	D	G	E	G	K	F	L	P				64
Bombyx mori chitinase		A	R	I	V	X	Y	F	S	N	W	A	V	Y	R	P	G										63
Human being chitotriosidase		A	K	L	V	C	Y	F	T	N	W	A	Q	Y	R	Q	G	E	A	R	F	L	P				59
Bovine cartilage gp-39		Y	K	L	V	C	Y	Y	T	S	W	S	Q	Y	R	E	G	D	G	S	C	F	P				50
Oviduct Specific Glycoprotein	SwissProt Q62010	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	45	
		Y	K	L	V	C	Y	F	T	N	W	A	H	S	R	P	G	P	A	S	I	M	P	H	D	L	56
	SwissProt P36718						27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	45	
							Y	F	T	N	W	A	H	S	R	P	G	P	A	S	I	L	P	H	D	L	55
	SwissProt Q28042						24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	42	
							Y	F	T	N	W	A	F	S	R	P	G	P	A	S	I	L	P	R	D	L	55
	SwissProt Q12889						27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	45	
							Y	F	T	N	W	A	H	S	R	P	G	P	A	S	I	L	P	H	D	L	55
Hen lysozyme		K	V	F	G	R	C	E	L	A	A	A	M	K	R	H	G	L	D	N	Y	R	G	Y	S	L	18

Fig. 6. NH_2-terminal amino acid sequence and alignment with the chitinase protein family.

that there is little homology with egg white lysozyme is in agreement with the finding that the 48.2 kDa chitinase does not have lysozyme activity. This may suggest that in animals the roles of chitinases and lysozymes are more specified.

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