In: Binomium Chitin-Chitinase: Recent Issues ISBN 978-1-60692-339-9
Editor: Salvatore Musumeci and Maurizio G. Paoletti

Chapter XX

Role of Chitinases in Human Stomach for Chitin Digestion: AMCase in the Gastric Digestion of Chitin and Chit in Gastric Pathologies

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Abstract

Chitin-containing food is an interesting but underestimated source of locally available, in most cases sustainable, food although chitin digestion by humans has generally been questioned or denied. Only in recent times chitinases have been found in several human tissues and their role has been associated with defence against parasite infections as well as with some allergic conditions. We reflected that crustaceans, and to some extent molluscs, mushrooms and most arthropods containing chitin, are sometime a consistent part of food regimes for local communities. Finally, we demonstrated that AMCase is present in gastric juices and it is associated with chitin digestion. In most tropical and some temperate countries, such as Japan and Korea, a significant number of adult insects and larvae are consumed raw, or cooked along with diverse local specialities. At present, up to 2,000 species of insects and other terrestrial arthropods have been listed as edible in Africa, Asia, Central and South America, Australia and Europe. Both insects and crustaceans are covered by chitin teguments and mushrooms contain some chitin. In most cases, the hard covering of polysaccharide chitin on insects accounts for 5-20% of their dry weight. In general, chitinases can digest chitin and reduce it to simple compounds such as *N*-acetyl-glucosamine. Western society does not

consider insects an important food, however: crustaceans, such as lobsters and crabs, are commonly eaten after discarding the hardened chitin-rich tegument, with the exception of small shrimps, which are generally eaten fried. Therefore, Western nutrition does not seem to depend on chitinases. These and other considerations, including the absence of chitin as a human body component, have led us to ask whether humans are capable of chitin digestion.

To assess chitinases' function as tools to digest chitin, we have examined 48 patient's gastric juices, obtained during gastroscopy, at Padova University Hospital. We found that 14.6% of total samples studied showed AMCase activity from 36.270 to 3.540 nmol/ml/h. The majority of involved subjects (75%) had lower values, from 2.800 to 0.178 nmol/ml/h; while in 10.4% of subjects the chitinolitic activity varied from 0.086 to 0.013 nmol/ml/h, and could be considered absent. We reported superficial digestion of fly forewings, utilizing gastric juice of a patient with an AMCase activity of 19.410 nmol/ml/h.

If AMCase enzyme, present in gastric juice, is truly involved in chitin digestion, we should expect a higher presence of expressed AMCase in populations currently accustomed to eating mushrooms and/or invertebrates bearing chitin.

We also found a positive relationship between *CHIT* expression level in antral gastric mucosa and both flogosis and *Helicobacter pylori* infection.

1. Introduction

Food containing some amount of chitin, such as crustaceans, molluscs, mushrooms and insects, is part of the human diet, especially for local communities in tropical and subtropical countries such as in Africa (Malaisse, 1997; Paoletti, 2005). This overlooked resource represents an important base for everyday survival, as well as a sustainable local resource available without the use of sophisticated traps or weapons even to the less empowered community members, like women and children (Dufour, 1987; Paoletti *et al.* 2000; Paoletti, 2005a) (Figure 1).

In most tropical and some temperate countries, such as Japan and Korea, a significant number of adult insects and larvae are consumed raw or cooked along with diversified local specialities. At present up to 2000 species of insects and other terrestrial arthropods have been listed as edible in Africa, Asia, Central and South America, Australia and Europe (Paoletti and Bukkens, 1997; DeFoliart, 2002; Paoletti, 2005).

Both insects and crustaceans are covered by chitin teguments. In most cases, the hard covering of polysaccharide chitin on insects accounts for 5-20% of the dry weight.

In general, chitinases can digest chitin and, usually in combination with other enzymatic activities, convert it into more readily absorbable components such as *N*-acetyl-glucosamine (Talent and Gracy, 1996; Jollès and Muzzarelli, 1999; Gardiner, 2000). Two kinds of chitinase have been reported with an exochitinase and endochitinase activity. The first releases *N*-acetyl-glucosamine from chitin on each occasion, while the second releases not *N*-acetylglucosamine, but instead a mixture of chitobiose and larger oligomers (Cohen-Kupiec and Chet, 1998; Fusetti *et al.* 2002; Tikhonov *et al.* 2004).

Figure 1. Edible invertebrates bearing chitin. A) Australian eating sweet ant (*Camponotus* sp.); B) Terrestrial spieders (*Theraphosa* sp.) eaten in Alto Orinoco (Venezuela); C) Caterpillars (*Erinnys ello*) are among the most appreciated insects in the Amazon; D) Many ants and termits are eaten in the tropics. Here the army ant *Eciton* sp. from Alto Orinoco; E) Small freshwater shrimpf raw or cooked are eaten in most areas included Amazon and Alto Orinoco.

Western society, interestingly, does not consider insects an important food (DeFoliart, 1999; DeFoliart, 2002), although crustaceans, such as lobsters and shrimps, are commonly eaten, albeit mostly after discarding the hardened chitin-rich tegument; although small shrimps are eaten with their teguments. Therefore, Western nutrition does not seem to depend on chitinases (DeFoliart, 1992). This and other considerations, including the absence of chitin as a human body component, have led some authors to question whether humans are capable of digesting chitin (Bukkens, 1997; Boot *et al.* 2005; Bukkens, 2005), and others still to suggest that its role is merely that of a dietetic fibre (Muzzarelli, 2001). Most nutritionists have argued the impossibility of humans feeding on and digesting chitin-containing food.

Evidence of chitinases in the human body is relatively recent: since 1994 for Chitotriosidase (Chit) and 2001 for Acidic Mammalian Chitinase (AMCase), (Hollak *et al.* 1994; Boot *et al.* 2001).

The function of human chitinases in diseases is still largely unknown. The only evidence available is that it occurs at high levels in certain disease states. For instance, Renkema *et al*. (1997, 1998) and Aerts (2009) in this book have described the occurrence in the plasma of patients affected by Gaucher disease of elevated levels of Chitotriosidase, a hydrolytic

enzyme produced by macrophage cells, which exhibits optimum activity at pH 6. More recently, it has been reported that Chit may also be involved in innate immune responses (Van Eijk *et al.* 2005). Moreover, Chit levels in plasma have been shown to be high in the case of diseases such as acute malaria (Barone *et al.* 2003), beta-thalassemia (Barone *et al.* 1998; Barone *et al.* 2001), and other hemoglobinopathies (see other chapters in this book) indicating that macrophage activation is responsible for Chit expression (Bouzas *et al.* 2003; Musumeci *et al.* 2005).

Another chitinase, Acidic Mammalian Chitinase (AMCase), produced in different tissues (Boot *et al.* 2001), exhibits optimum activity in the acid pH range and recently has been implicated in allergic bronchial asthma (Zhu *at al*., 2004). To date, however, its function is obscure (Boot *et al.* 2005). AMCase has also been found in mouse and rat stomachs, where it has been shown at the cellular level (immunohistochemically) and at the level of RNA expression (Suzuki *et al.* 2002; Goto *et al.* 2003; Boot *et al.* 2005;).

Considering that chitinases are highly conserved in mammals (from humans to rats) (Gianfrancesco and Musumeci, 2004), it is to be expected that also humans could produce AMCase in the gastric epithelium, where it would digest chitin from parasites and food, including arthropods (insects and crustaceans) and mushrooms containing some chitin.

Boot *et al*. showed in 2005 that AMCase is expressed in the human stomach and, to a lesser extent, in the lung; but its presence and chitinolytic activity in human gastric juice have been confirmed only recently (Paoletti *et al.* 2007).

This chapter aims: a) to analyse AMCase activity in human gastric juice; b) to assess whether humans are able to digest the cover of chitinous arthropods (including insects) by analysing digestion of chitinous insect wings by gastric juices; c) to show the results of expression of *CHIT* and *AMCase* in human gastric mucosa in relation to stomach pathologies such as phlogosis and *Helicobacter pylori* infection (Figure 2).

2. Sampling Chitinases in Gastric Juices

At Padova University Polyclinic, 48 Italian subjects (26 males and 22 females), aged 23-76 years were submitted to gastroscopy because of symptoms of dyspepsia and postprandial pains (Table 1). All subjects lived on the outskirts of Padova. The study was conducted between April 2006 and July 2007. The patients consumed no food for at least 14 hours prior to the gastroscopy.

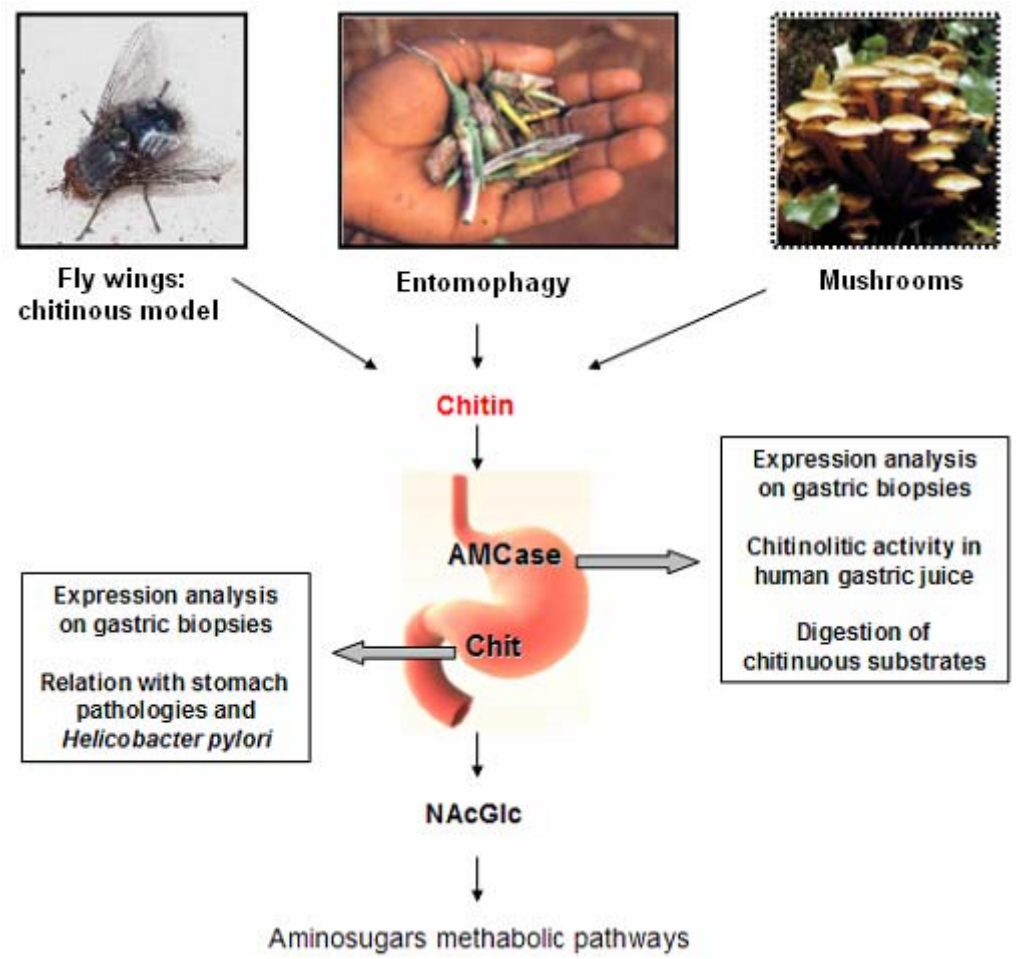

Figure 2. Schematic description of the aims of this chapter.

After the optical examination of the gastric mucosa a modified Giemsa test for detection of *Helicobacter pylori* (detected as a Giemsa positive bacillus) was also performed on all the patients. According to the Sydney Sistem (Dixon *et al.* 1996), the gastritis score relating to each biopsy site was then evaluated using a visual analog scale (+ mild; ++ moderate; +++ severe).

A fragment of gastric mucosa was collected for immunohystochemical analysis and to measure mRNA for *CHIT* and *AMCase* by Quantitative Real Time PCR. No drugs that could potentially interfere with the gastric juice secretion were administered before the gastroscopy. Before removing the gastroscope, ~ 10 ml of gastric juice were collected from each subject. Samples were held in ice until they were transferred to the Department of Biology at the University of Padova, where they were rapidly frozen at -80°C. Once the collection of gastric juice was completed, the frozen samples were transported in dry ice to the Department of Pediatrics at the University of Catania, where they remained frozen at -80°C until the determination of chitinase activities. The study was approved by the Ethics Committees of the University of Padova.

Table 1. Patient's personal data (sex and age), AMCase activity in gastric juice measured with the spectrofluorimetric method developed by Boot et al. expressed as nmol/ml/h (Boot, 2001), gastroscopic diagnosis and results of *Helicobacter pylori* test of each patient envolved in our studies (Paoletti *et al.* 2007; Cozzarini, 2007)

Subject No.	Sex M - F	Age (years)	AMCase activity (nmol/ml/hr)	Gastroscopic diagnosis
1	M	73	0.400	No affection
2	F	53	3.540	No affection
3	F	74	6.800	Chronic antral gastritis and gastric micropolyposis
4	F	58	36.270	No affection
5	F	59	0.260	Chronic antral gastritis, suspected Short Barrett esophagitis
6	M	64	0.086	Reflux disease
7	M	43	0.025	Chronic antral gastritis and duodenitis
8	M	45	0.065	Chronic gastritis
9	M	40	0.013	Antral gastritis
10	F	50	0.046	No affection
11	F	65	4.520	Antral gastritis, *H. pylori* test positive
12	F	39	0.178	Antral gastritis and reflux disease
13	M	74	0.256	Antral gastritis, reflux disease, *H. pylori* test positive
14	F	60	0.446	Slight antral gastritis
15	F	52	0.330	Antral gastritis and leiomioma, hiatal hernia
16	F	53	0.303	Antral gastritis, reflux disease, *H. pylori* test positive
17	M	50	0.299	Antral gastritis, reflux disease, *H. pylori* test positive
18	M	51	0.558	Antral gastritis, reflux disease, *H. pylori* test positive
19	M	62	19.410	Diffuse gastritis and reflux disease
20	M	48	0.249	Antral gastritis, reflux disease, *H. pylori* test positive
21	M	45	0.480	Antral gastritis, reflux disease, *H. pylori* test positive
22	F	54	0.549	Antral gastritis, reflux disease, gastric micropolyposis
23	M	46	0.181	Antral gastritis and reflux disease
24	M	40	0.230	Antral gastritis, reflux disease, *H. pylori* test positive

Subject No.	Sex M - F	Age (years)	AMCase activity (nmol/ml/hr)	Gastroscopic diagnosis
25	M	60	0.210	Diffuse gastritis, reflux disease, *H. pylori* test positive
26	F	62	0.252	Diffuse gastritis, reflux disease, *H. pylori* test positive
27	F	25	0.231	Reflux disease and suspected Short Barrett esophagitis
28	M	71	0.252	Chronic antral gastritis, reflux disease and duodenitis
29	F	44	0.249	Slight reflux disease
30	M	73	27.990	Slight reflux disease
31	F	76	2.438	Reflux disease, gastric micropolyposis, *H. pylori* test pos.
32	F	23	0.267	Chronic antral gastritis, reflux disease and metaplasia
33	M	35	0.350	Reflux disease, duodenitis, *H. pylori* test positive
34	M	69	0.370	Chronic antral gastritis and reflux disease
35	M	60	0.350	Gastric micropolyposis
36	M	66	0.510	Slight gastritis and duodenitis
37	M	73	14.500	Chronic antral gastritis, reflux disease, gastric micropolyposis
38	M	60	0.300	Chronic antral gastritis
39	F	40	0.230	Diffuse gastritis, reflux disease and esophageal candidiasis
40	F	71	0.270	Diffuse gastritis and reflux disease
41	M	35	0.250	Antral gastritis and reflux disease
42	F	55	0.370	Reflux disease and slight congestive gastropathy
43	F	74	0.250	Chronic antral gastritis and gastic micropolyposis
44	F	58	0.250	Slight gastritis, reflux disease and esophageal candidiasis
45	M	50	2.800	Chronic antral gastritis and reflux disease
46	M	46	0.300	Slight gastritis and reflux disease
47	M	75	0.400	Antral gastritis and slight reflux disease
48	F	25	0.200	Chronic antral gastritis

2.1. Preparation of Gastric Juice for AMCase Activity Determination

The tubes containing the gastric juice were left to defrost at 4°C and centrifuged for 30 minutes at 15,000 *g* at 4°C. The supernatant was aspired and fractioned into 1 ml Eppendorf tubes and stored at -80°C until examination.

2.2. AMCase Activity

We used the spectrofluorimetric methodologies developed by Boot et al. (Boot *et al.* 2001) and Tikhonov et al. (Tikhonov *et al.* 2004). Since these two methods are comparable, here we report only the results related to the first one (the Boot *et al*. method).

After the gastric samples were defrosted the pH was measured and corrected to pH 2 with 0.1 M HCL The value of pH 2 was chosen because it is the optimum pH for the dosage of AMCase (Talent and Gracy, 1996). Fifty µl of gastric juice were incubated with 0.1 ml of a solution containing 22 mmol/l of the artificial substrate 4-methylumbelliferyl-β-D-N,N'-diacetylchitobiose (Sigma Chemical Co catalogue M 9763) in 0.5 M citrate-phosphate buffer pH 4.5 for 30 minutes at 37°C. The reaction was stopped by using 2 ml of 0.5 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 10.7. The fluorescence was read by a spettrofluorimeter Hitachi 2500 (Hitachi, Europe Ltd, Herts, UK), on 365 nm excitation and 450 nm emission. AMCase activity was expressed as nanomoles of substrate hydrolyzed per ml per hour (nmol/ml/h). A blank for control, composed of reagents (0.5 M citrate-phosphate buffer + 4-methylumbelliferyl-β-D-N,N'-diacetylchitobiose) was used in each measurement and two standard samples at different chitinase activity (10 and 100 nmol/ml/h) were also added. The interassay variation coefficient (reproducibility) was <5%. The reported results are the mean of three determinations and the graphic representations are obtained by using the Prisma software. Median and range were calculated for each group.

2.3. Inibition Test with Allosamidin

To confirm that the hydrolytic activity was due to AMCase the dosage was repeated after neutralisation of gastric juice chitinase activity with 9 µM of allosamidin (kindly provided by Dr S. Sakuda, Sakuda and Sakurada, 1998), for 90 minutes at 37°C in a shaking water bath (160 rpm). This concentration is likely to completely inhibit chitinase activity (Jollès and Muzzarelli, 1999). The neutralisation reaction was stopped by adding 180 µl of sodium dodecylsulfate (DS) 10% wt/vol.

2.4. Chitinolytic Activity at Different pH Values

Samples of gastric juice initially at pH value > 6 were adjusted at different values decreasing of one unit with 0.1 M HCL and the chitinolytic activity was measured in each sample with the Boot *et al* 2001 method.

The activity of each sample measured by fluorescent emission was stable after repeated measurements. The operator's great experience and the simultaneity of determinations excluded processing artefacts.

3. Results

Among 48 studied patients, 4 (patients 1, 2, 4, 10), who at physical and clinical examination were otherwise healthy resulted negative for gastritis and they represented the healthy controls. The majority of patients (38/48) showed acute or chronic gastritis and gastric micropoliposis, while 32/48 patients showed signs of gastroesophageal reflux disease (GERD) and more in general dyspeptic symptoms and postprandial pain. Only 12/48 patients tested resulted positive for *Helicobacter pylori* (Table 1).

Table 1 reports the chitinase activity of each gastric sample, reported in nmol/ml/h of 4-methylumbelliferyl-beta-D-N,N′-diacetylchitobiose hydrolised.

In Table 2, the patients were assigned to one of three groups according to the values of AMCase activity. 14.6% of total studied samples showed AMCase activity from 36.270 to 3.540 nmol/ml/h, the majority of involved subjects (75%) had lower values from 2.800 to 0.178 nmol/ml/h, while in 10.4% of subjects the chitinolitic activity varied between 0.086 and 0.013 nmol/ml/h and could be considered absent. The prevalence of *Helicobacter pylori* was distributed among the three group examined. No statistically significant correlation was found between the AMCase activity and the sex or age of patients.

In the two control samples, where no gastric juice was added, the level of activity was < 0.1 nmol/ml/h. The standard samples added in each determination confirmed the reproducibility of the method with a variation coefficent of < 5% .

In 11 separate samples, after neutralisation of chitinase activity with allosamidine the AMCase activity disappeared, confirming that the results are due specifically to the chitinolytic activity of gastric juice (Figure 3).

The curve of chitinase activity in function of the pH demonstrated that the chitinolytic activity was high at pH 2, was stable up to pH 5-6 and decreased slowly to a value of pH > 6, confirming that chitinolytic activities obtained at different pH always belong to AMCase (Figure 4), similarly to mouse AMCase.

Table 2. Chitinolytic activity (AMCase) at pH 2 expressed in nmol/ml/h of hydrolyzed substracts (Paoletti *et al.* 2007)

Patients	%	AMCase activity (nmol/ml/h)	Age	Sex M/F
7	14.6	16.147 (36.270 - 3.540)	65 (53 - 74)	3/4
36	75	0.439 (2.800 - 0.178)	53 (23 - 76)	19/17
5	10.4	0.047 (0.086 - 0.013)	48 (40 - 64)	4/1

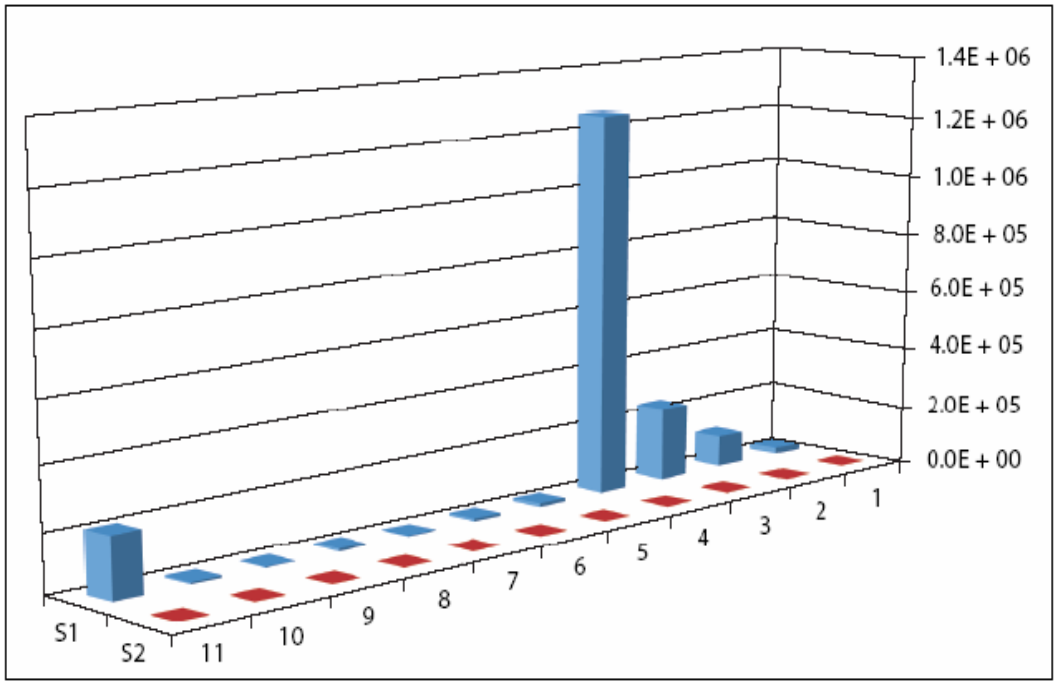

Figure 3. FITC-chitin activity expressed in fluorescence emission (CPS) before (S1) and after (S2) inhibition with allosamidin (9 µM), in the first 11 subjects (Paoletti *et al.* 2007).

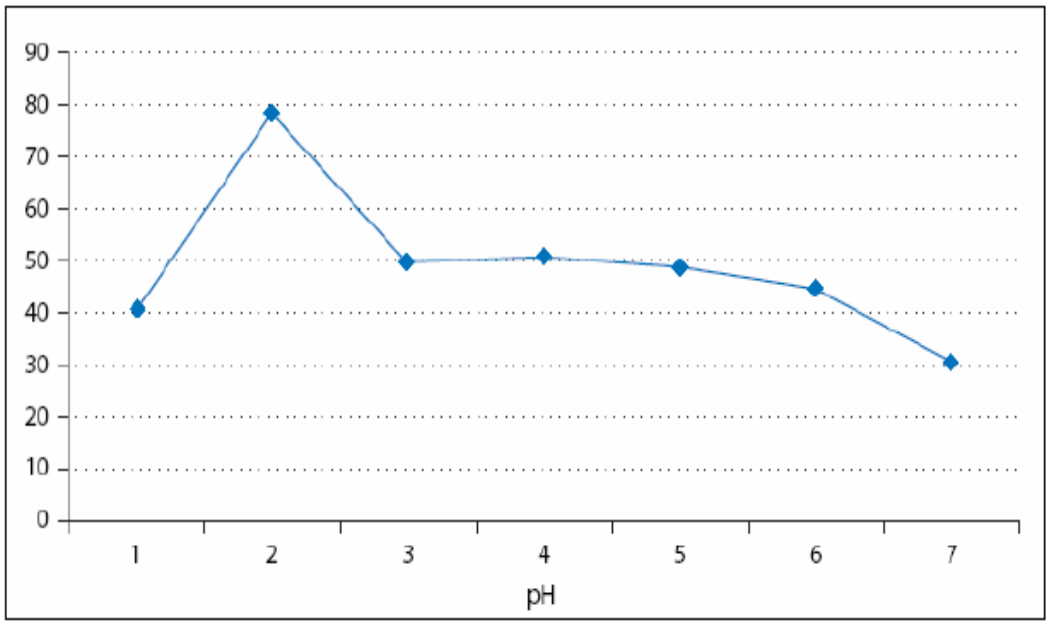

Figure 4. AMCase activity (nmol/ml/h) of gastric juice of subject 4 at different pH values (Paoletti *et al.* 2007) .

4. Is Chitin Digestion Really Affected by Chitinase Presence in Gastric Juice?

We performed microscopic observations of chitin degradation, utilising fly wings as chitinous substrates (Karelina, 2007) and the gastric juice of a patient having an AMCase activity of 19.41 nmol/ml/h. After immersion at 37°C for 4 hours of fly forewings (obtained from *Calliphora vomitoria* L. and *C. vicina* R.-D.) in human gastric juice, we assessed morphological changes using a straight microscope (Leica DMR, software IM500).

Figure 5 shows the damage caused by the gastric juice treatment after 2, 4 and 6 hours (Figures 5b-d), compared with a control wing processed with HCl 1 M at pH 2, in which the surface morphology appears intact (Figure 5a).

Figure 6 shows: microscopic observations (stereomicroscope Leica MZ16, software IM500) of chitin degradation after 8 hour treatment with human gastric juice, caused by the chitinase activity, on the border of two whole wings (Figures 6a and 6c) and a particular of these wings (Figures 6b and 6d); the effect of gastric juice after 8 hours treatment on wing's surface (Figure 6e) at ESEM microscope (ESEM XL30, software XL30); the particular of a wing (Figure 6f) treated only with HCl 1 M at SEM microscope (CAMBRIDGE STEREOSCAN 260, software Matrox Intellicam v. 2.0).

The chitinase effects on wing integrity, as judged by morphological examination, appear rather superficial, and could be considered as evidence of digestion due to AMCase presence in gastric juice. Since no modification was observed incubating the wing with HCl 1 M, the only possible conclusion is that modification of fly wings could be the effect of gastric juice. However, when we measured the amount of chitin metabolite in the gastric juice, where the fly wings were incubated, the *N*-acetylglucosamine (GlcNac) content, measured in solution, was very low. This result was not surprising, because the main product of chitin breakdown after incubation with endochitinase is a mixture of chito-oligomers $GlcNAc_n$ with n >2 and chitobioside (n=2). In our previous article (Paoletti *et al.* 2007) we demonstrated that in humans the gastric chitinase functions differently as an endochitinase from the other chitinases present in nature. In fact, when we varied the incubation time from 30 to 120 minutes the fluorescence intensity of FITC-chitin increased rapidly, reaching values from 200,000 to 1,400,000 CPS (data not shown). This suggests that hydrolysis starts randomly in the middle of the FITC-chitin polysaccharide chain, generating fluorescent fragments. On the contrary exochitinase activity, typical of bacterial chitinases (Fusetti *et al.* 2002), is characterised by a slow increment of fluorescence, since the hydrolysis takes place at the terminal of chitin molecules.

We exclude the possibility that gastric chitinase activity in our patients could be associated with food residues, since the patients did not consume food for at least 14 hours prior to the gastroscopy; and we are certain that the chitinases present are not produced by gastric flora, since the several species present in gastric juice secrete exochitinases and not endochitinase (Fusetti *et al.* 2002). This is also confirmed by extremely low *N*-acetyl-glucosamine content after incubation with fly wings.

As negative control we treated gastric juices with allosamidin in order to inhibite chitinase activity (Sakuda and Sakurada, 1998).

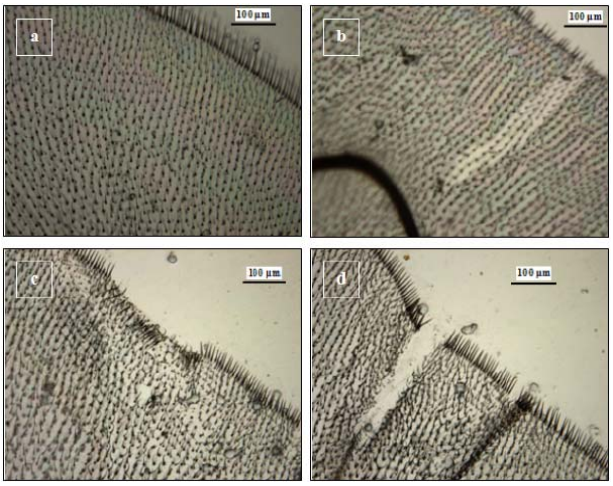

Figure 5. Microscopic observations with the straight microscope (Leica DMR, software IM500) of chitin degradation after 2 (b), 4 (c) and 6 (d) hour treatment with gastric juice of patient n°19 (19.410 nmol/ml/h), utilizing fly wings (C. vomitoria) as chitinous substrate. Damage probably caused by the chitinase activity (b, c, d) compared with blank wing processed with HCl 1 M (a). (Images from Kira Karelina, Thesis, University of Padova, 2007).

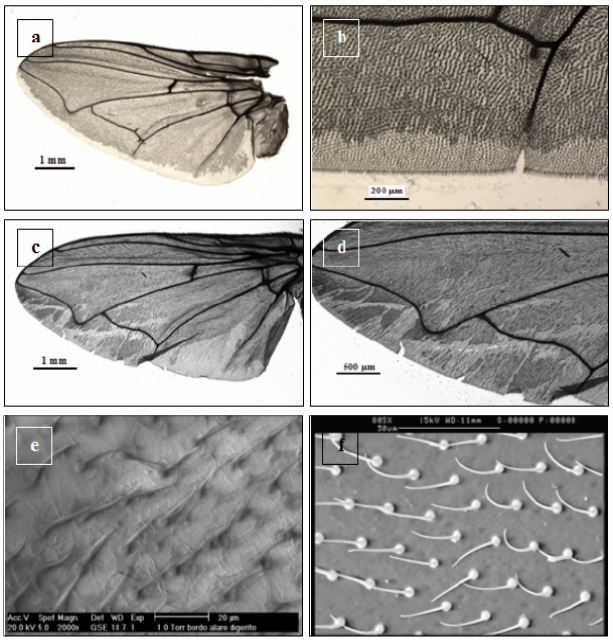

Figure 6. Microscopic observations of chitin degradation after 8 hour treatment with human gastric juice utilizing fly wings (*C. vomitoria*) as chitinous substrate. Vision at the stereomicroscope (Leica MZ16, software IM500) of damages, probably caused by the chitinase activity, on the border of two whole wings (a and c) treated with gastric juice and a particular of these wings (b and d). Effect of gastric juice after 8 hours treatment on wing's surface (e) at ESEM microscope (ESEM XL30, software XL30). Particular of a wing (f) treated only with HCl 1 M at SEM microscope (CAMBRIDGE STEREOSCAN 260, software Matrox Intellicam v. 2.0). (Images from Andrea Alfieri, Thesis, University of Padova, 2008).

In fact digestion blockage has been demonstrated because we observed no production of *N*-acetyl-glucosamine. We infer that chitinase and not other potential enzymes are involved in wings digestion.

Since the major visible effect is the softening and corrosion of the margins of wings, we think, based on these observations, that AMCase function in gastric juice may be to provide seasoning rather than extended fragmentation of the chitinous cover, permitting, however, a better digestion of the internal contents of chitin-covered organisms like caterpillars, insect larvae or adults.

5. Analysis of CHIT and AMCase Gene Expression with Qrt-PCR in Human Gastric Mucosa Biopsy

Stomach biopsies (antral mucosa specimens collected during gastroscopy on Italian patients at Padova University Hospital) from 27 patients have been studied for *CHIT* (Figure 7) and *AMCase* (Figure 8) gene expression with QRT-PCR in human gastric antral mucosa (Cozzarini, 2007; Cozzarini *et al.* 2008).

5.1. Total RNA Extraction and Quantification

Tissue samples were homogenised in 1 ml of Trizol® reagent (Invitrogen) using a power homogenizer (Ultra-turrax-T8; IKA® WERKE). After incubation of the homogenised samples for 5 min at room temperature, 200 µl of chloroform was added. Samples were mixed vigorously and held in ice for 15 min, centrifuged for 15 min at 16,100 *g* at 4°C. The RNA was precipitated from the aqueous phase by adding an equal volume of isopropanol. Then RNA was dissolved in RNase-free water.

For each sample, total RNA concentration was determined with spectrophotometrical analysis with NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

The quality of each sample total RNA was measured with the Agilent 2100 BioAnalyzer System (Agilent Technologies). Only good quality RNA was used for further experiments.

5.2. Quantitative Polymerase Chain Reaction

cDNA was prepared from 1 µg of single patient total RNA using Superscript II (Invitrogen) and oligo (dT), following manufacturer's instruction.

Quantitative real-time PCR (QRT-PCR) based on the SYBR™ Green chemistry (Applied Biosystems, Foster City, CA) was carried out to test the expression level of *CHIT* and *AMCase*. 50 ng of cDNA reverse-transcribed from patient's total RNA was amplified using DyNAmo HS SYBR Green qPCR Kit (Finnzymes).

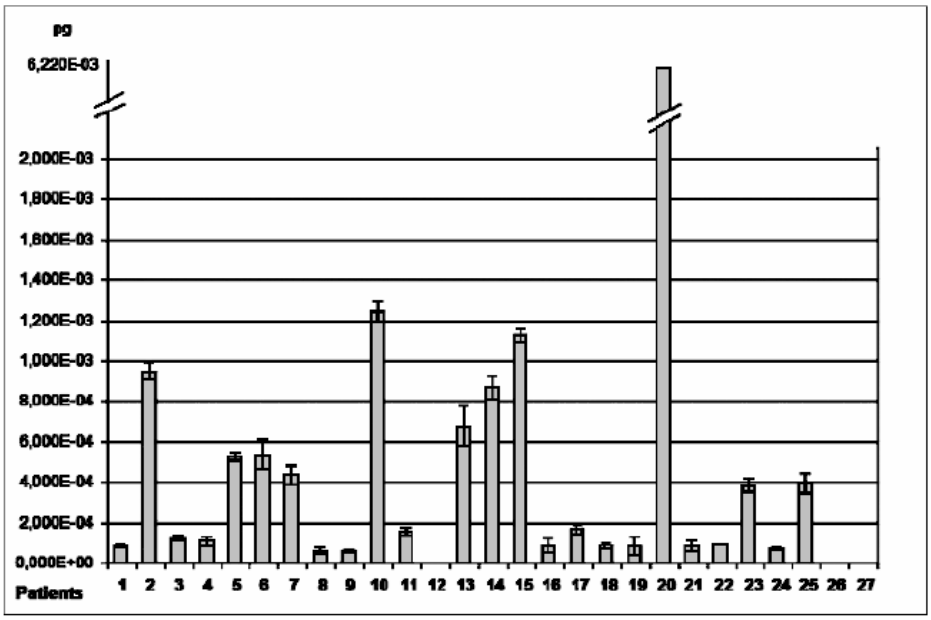

Figure 7. Quantitative real-time PCR analysis of Chitotriosidase (CHIT) mRNA in stomach biopsies from 27 patients. Patients 12, 26 and 27 showed no CHIT expression, while the CHIT-specific mRNA mean quantity was 5.399*10-4 ± 4.887*10-5 pg. Absolute expression levels are shown in mRNA pg, based on a standard curve. Standard Deviations (SD) calculated on the experimental replicates are also shown. (Figure from Elisa Cozzarini Thesis, University of Padova, 2007).

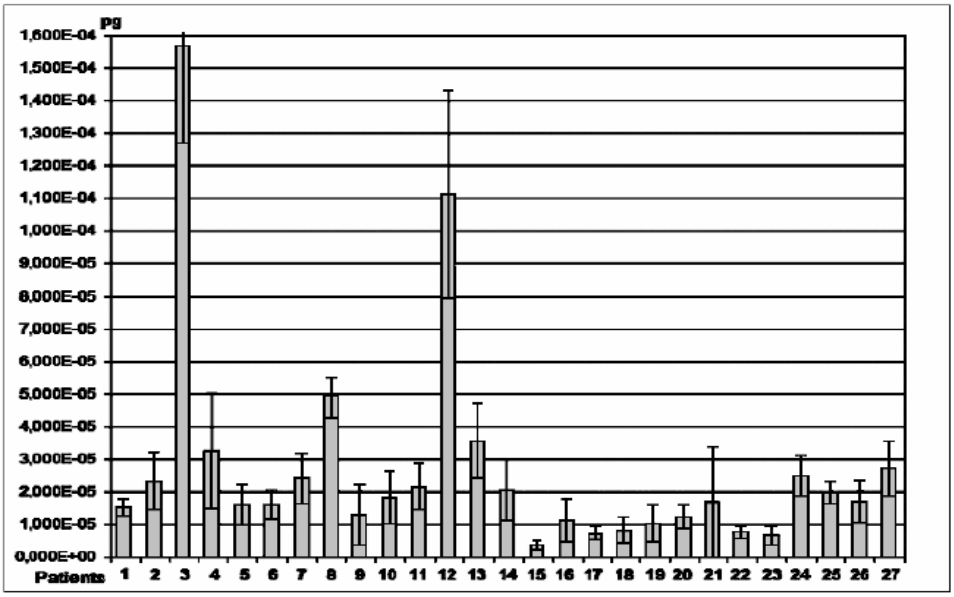

Figure 8. Quantitative real-time PCR of Acidic Mammalian Chitinase (AMCase) in stomach biopsies from 27 patients. Absolute expression levels are shown in mRNA pg, based on a standard curve. Standard Deviations (SD) are also shown. (Figure from Elisa Cozzarini Thesis, University of Padova, 2007).

PCR reactions were performed in a GeneAmp 9600 thermocycler, coupled with a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Gene-specific oligonucleotides were designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi).

CHIT primer sequences were designed according to the deposited mRNA ref-sequence (GenBank Accession No. NM_003465). *CHIT* primer sequences were 5'-GGGATGCTGGCCTACTATGA-3' (forward) and 5'-TAGGGCACCTTCTGATCCTG-3' (reverse). Primer sequences for *AMCase* were designed on the basis of the two deposited transcript variants (GenBank Accession NM_021797 and NM_201653). *AMCase* primer sequences were 5'-CTACGACCTCCATGGCTCCT-3' (forward) and 5'-TGCTCCATTGTCCTTCCAGT-3' (reverse) and were chosen to specifically identify both *AMCase* transcript variants.

In order to quantify *CHIT* and *AMCase* expression, we used the absolute quantification method, comparing patient samples to a standard curve (Bustin, 2000). The standard curves for each gene were constructed with five serial ten-fold dilutions of a purified and quantified PCR product (obtained with the same primers used for the qRT-PCR), with a sensitivity range of $7.4*10^{-2}$ pg – $7.4*10^{-5}$ pg (*CHIT*) and $3.8*10^{-2}$ pg – $3.8*10^{-6}$ pg (*AMCase*). Threshold cycles (Cts) obtained with patient's samples were compared to Cts generated from the specific standard curve. Results were expressed in quantity (pg) of specific amplified nucleic acid.

The equation used to calculate the pg quantity of template (mRNA) that was present at the beginning of the qRT-PCR reaction was: $pg = 10^{(Ct - b)/a}$, where a: the angular coefficient (slope) and b: the intercept.

Since the two genes we studied (*CHIT* and *AMCase*) were expressed at a very low level in the gastric mucosa, a classic qRT-PCR reaction (40 cycles) was not able to quantify the initial mRNA quantity. We then decided to stop the qRT-PCR reaction during the linear phase (i.e. after 10 amplification cycles), to add new mix (master mix and half concentration of primers) and to start with a second classic qRT-PCR reaction (40 cycles). The linearity of the reaction was preserved and the sample Cts fell in the standard curve sensitivity range.

Three replicates for each patient cDNA were performed, and mean and SD were calculated.

The correlation of the *CHIT* and *AMCase*'s expression level in human gastric mucosa with the severity of stomach inflammation and the *H. pylori* infection was analysed by the χ^2 test and significant P value < 0.05 was accepted.

6. Results

CHIT was expressed at low levels in the stomach of all the studied individuals except three patients who showed no expression (Figure 7). In particular, *CHIT* mRNA quantity varied between $6.315*10^{-5}$ pg to $6.220*10^{-3}$ pg, with a mean quantity of $5.427*10^{-4} \pm 4.703*10^{-5}$ pg.

Quantitative real-time PCR confirmed the presence of *AMCase* mRNA in human gastric mucosa, even though this gene was expressed at a very low level (Figure 8). In particular the

majority of our patients examined (19/27) showed *AMCase* mRNA quantity between $1.014*10^{-5} \pm 2.432*10^{-6}$ pg and $3.529*10^{-5} \pm 7.815*10^{-6}$ pg. In 5 patients *AMCase* was weakly expressed (about 10^{-6} pg). Three patients had the highest *AMCase* mRNA expression corresponding to a mean value of $1.056*10^{-4} \pm 7.592*10^{-5}$ pg.

This research indicated an interesting positive correlation between *CHIT* expression and gastric inflammation (P value= 0.026) and *Helicobacter pylori* infection (P value= 0.016). We did not observe any positive correlation between AMCase activity in gastric juice and mRNA expression in gastric mucosa. This could be due to the complexity of the human stomach tissue, which has different biological attitudes in fundus, corpus and antrum portions.

Our study has demonstrated for the first time that *CHIT* mRNA is also present in gastric mucosa and this result could represent further evidence of the involvement of Chitotriosidase enzyme in human immune response (Cozzarini *et al.* 2008).

7. Discussion

In this study, we demonstrated AMCase activity in human stomach associated with chitin digestion and possibly with digestion of chitin-containing foods such as invertebrates and mushrooms. The function of protection from gastric parasites is another possibility. The fraction of the population (14.6% in Caucasian, Padova-living peoples studied) having high activity of AMCase in the stomach could have more potential for chitinase digestion. No one has, to our knowledge, looked at the link between AMCase genotypes and intestinal parasites, but if we want to put forward the hypothesis that gastric AMCase can protect against parasitic helmints, we need to consider that many parasites only have a very short gastric phase, but have a longer developmental phase in the lung (*Ascaris lumbricoides*), where AMCase could also play a role. On the contrary AMCase or macrophagic Chit present in the stomach may possibly better protect from parasites such as nematodes like *Ascaris* and flatworms like *Taenia*, because the eggs of both have some chitin cover (Wimmer *et al.* 1998; Harter *et al.* 2003). Chitin is definitely present in the egg-shells of *Ascaris lumbricoides* (Sromová and Lýsek, 1990), but it is doubtful whether external chitinases can access it at all. Since the eggs are produced in the intestine where the adult worms reside, so they are not in contact with gastric chitinases. Only for ingested Ascaris or *Taenia* eggs, therefore, could this protective mechanism be operative.

It is probable that the macrophage cells infiltrating gastric mucosa of patients who had gastroscopy for clinical symptoms of gastritis produced Chit in response to the inflammation, with an optimum at pH 5.2 or higher. In fact, we found a high correlation between *CHIT* mRNA expression and *Helicobacter pylori* infection (P value = 0.016). Phlogosis and *CHIT* mRNA have also shown consistent correlation (P value= 0.026).

Chitotriosidase apparently has similar effects on chitin, but the *CHIT* expression in gastric mucosa respond quickly to inflammation and *Helicobacter pylori* infection as a consequence of macrophages activation.

Even if the potential to digest chitin has been documented in 43/48 (i.e. 89.6%) of our Italian patients, we do not know the minimum quantity of chitinases needed to effectively digest the chitin associated with insects and crustaceans.

Food bearing chitin can, possibly, be better digested by peoples having higher rate of chitinase expression. Our current experimental evidence on fly forewings suggest chitinases have a role in the seasoning and shallow digestion of this wing substrate. We infer that chitinases may act as complements in breaking down chitinous cuticles and allowing other enzymes to better utilise invertebrates' or mushrooms' content. However, more work is needed to elucidate these steps in greater detail, including a more general view of chitin digestion in animals not limited to humans.

Do people whose diet relies on chitinous food have a different gut flora from people who do not? in other words, does the gut flora of entomophagous people contain bacterial populations where chitinolytic bacteria are more prevalent? An example could be the relation between termites and cellulosa or bacterial chitinases found in the stomach of fish, or the occurrence of several chitinolytic bacterial strains in the gut of entomophagous bats (Alwin Prem Anand and Sripathi, 2004).

Population-epidemiologic studies are needed to assess different populations in adopting different food resources containing and not containing chitin.

Chitin has a very long history in animal and human evolution, and chitinases have coevolved within different lifestyles as evolutionary consideration in several parts of this book seem to demonstrate.

The higher chitinase activity in tropical human populations with higher rates of entomophagy could represent an adactative response to alimentary habits, conferring increased resistance against parasitic infection in these areas and faciliting the digestion of chitin through bacterial chitinases. This view, however, needs to be more carefully substantiated.

Acknowledgments

We are greately indebted to Franco Falcone for discussion of several points of our work. Andrew Baldwin has provided assistance with manuscript review. Many thanks to Kira Karelina and Andrea Alfieri for the fly forewing's photos.

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