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Human Gastric Juice Contains Chitinase That Can Degrade Chitin

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Key Words
Acidic mammalian chitinase · Gastric juice · Chitin digestion · Entomophagy

Abstract
Chitin digestion by humans has generally been questioned or denied. Only recently chitinases have been found in several human tissues and their role has been associated with defense against parasite infections and to some allergic conditions. In this pilot study we tested the gastric juices of 25 Italian subjects on the artificial substrates 4-methylumbelliferyl-β-D-N,N′,diacetylchitobiose or/and fluorescein isothiocyanate (FITC) chitin to demonstrate the presence of a chitinase activity. Since this chitinase activity was demonstrated at acidic pH, it is currently referred to acidic mammalian chitinase (AMCase). AMCase activity was present in gastric juices of twenty of 25 Italian patients in a range of activity from 0.21 to 36.27 nmol/ml/h and from 8,881 to 1,254,782 fluorescence emission (CPS), according to the used methods. In the remaining five of 25 gastric juices, AMCase activity was almost absent in both assay methods. An allosamidine inhibition test and the measurement at different pH values confirmed that this activity was characteristic of AMCase. The absence of activity in 20% of the gastric juices may be a consequence of virtual absence of chitinous food in the Western diet.

Introduction

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 2,000 species of insects and other terrestrial arthropods have been listed as edible in Africa, Asia, Central and South America and Australia [1–3]. Both insects and crustaceans are covered by chitin teguments. In most cases, the hard cover polysaccharide chitin of insects accounts for 5–20% of the dry weight. In general, chitinases can digest chitin and reduce it into assimilable components such as N-acetyl-glucosamine [4–6]. Western society does not consider insects an important food [7, 2]; however, crustaceans such as lobsters and shrimps are commonly eaten mostly after discarding the hardened chitin-rich tegument. Therefore, Western nutrition apparently does not seem to depend on chitinases [8]. This and other considerations, including the absence of chitin as a human body component, have led some authors to ask whether humans are capable of digesting chitin [9–12] and others to suggest its role as dietetic fibers [13].

Even the function of human chitinases in disease is still largely unknown. The only evidence available is that it is found in high levels in certain disease states. For instance, Renkema et al. [14, 15] have described, in plas-

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ma of patients affected by Gaucher disease, elevated levels of chitotriosidase (Chit), a hydrolytic enzyme produced by macrophage cells, which exhibit optimum activity at pH 6. More recently it has been reported that Chit may also be involved in innate immune responses [16]. Moreover Chit has been shown to be high in diseases such as acute malaria [17], beta-thalassemia [18, 19], and other hemoglobinopathies, indicating that macrophage activation is responsible for Chit expression [20, 21].

Another chitinase, acidic mammalian chitinase (AMCase), produced by the bronchial epithelium, exhibits optimum activity in the acid pH range and has been implicated in allergic bronchial asthma [22]. To date, however, its function is obscure [9]. AMCase has also been found in the mouse and rat stomach where it has been shown at the cellular level (immunohistochemically) and at the level of RNA expression [9, 23, 24].

Considering that CHIT gene sequences are conserved in human, mouse and rat [25], it is expected that humans could produce AMCase in the gastric epithelium, where it would digest chitin from food, including arthropod insects, whose cuticle is made of chitin [12]. Boot et al. 2005 [9] have shown that AMCase is expressed in the human stomach, and to a lesser extent in the lung, but its activity in human gastric juice has not yet been reported.

This research aims to determine whether AMCase activity is present in human gastric juice and to asses whether humans are able to digest the cover of chitinous arthropods (including insects).

Materials and Methods

At Padua University Polyclinic 25 subjects (14 males and 11 females), aged 39–74 years, were submitted to gastroscopy because of symptoms of dyspepsia and postprandial pains. All subjects lived in the outskirts of Padua (originally from Padua except for one subject from the Philippine Islands; patient n = 17). The study was conducted in the period between March and April 2006. A detailed physical and clinical examination before the gastroscopy showed that 4 subjects were otherwise healthy. The patients consumed no food for at least 12 h prior to the gastroscopy. After the optical examination of the gastric mucosa a ^{13}C-urea breath test for detection of *Helicobacter pylori* was also performed on all the patients according to the Hilker et al. [26] 1996 method. A fragment of gastric mucosa was collected for immunohistochemical analysis. No drugs which could interfere with the gastric juice secretion were administered before the gastroscopy. Before removing the gastroscope, 10 ml of gastric juice was collected from each subject. Samples were held in ice until they were transferred to the Department of Biology at the University of Padua, 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 local Ethical Committees of the University of Padua.

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 min 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.

AMCase Activity

We used the spectrofluorimetric methodologies developed by Boot et al. [27] and by Tikhonov et al. [28]. 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 [27].

AMCase Activity [27]. 50 μl of undiluted 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 min 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 spectrofluorimeter Hitachi 2500 (Hitachi, Europe Ltd., Herts, UK), at 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 coefficient of variation (reproducibility) was <5%. The reported results were the mean of three determinations.

AMCase Activity [28]. Fluorescein isothiocyanate (FITC)-chitin was prepared by Prof. Luis V. Lopez-Llorca, University of Alicante (Spain) using Chitosan (Sigma) and fluorescein isothiocyanate (FITC, Sigma). The degree of substitution was calculated from absorbance of FITC-chitin solution in 0.5% (v/v) acetic acid solution at 490 nm and an extinction coefficient of 1.22×10^6 M cm^{-1}. The labeled FITC-chitin after lyophilization appears as a yellow-orange powder. 6 mg of FITC-chitin were dispersed in 5 ml of 0.05 M Tris-HCl (pH 8.5) buffer and the mixture was kept at room temperature for 1 h. With the aim to remove all unlabelled FITC, the solution was then filtered using Minisart single-use filter (0.20 μm) (Sartorious, UK) and the filtered liquid was collected in a cuvette (3 ml and 10 mm width) and read in a Spex Fluorolog-2 (model F-111) spectrofluorimeter, using an excitation wavelength of 490 nm, and the measurement was performed at 513 nm, at a maximum FITC emission. This procedure was repeated four times until all fluorescence not linked to chitin was eliminated. Once the FITC-chitin substrate had been washed, it was collected inverting the flux of washing liquid and 100 μl of FITC-chitin in 0.05 M Tris-HCl (pH 8.5) buffer was mixed with 0.1 ml of gastric juice pH 2. The mixture was incubated for 60 min at 37 °C in a shaking water bath (160 rpm). The mixture was then added to 3 ml of 0.5 M Tris-HCl (pH 8.5) and filtered. The fluorescence of the filtrate was read using an excitation wavelength of 490 nm and an emission wavelength of 513 nm. The intensity of

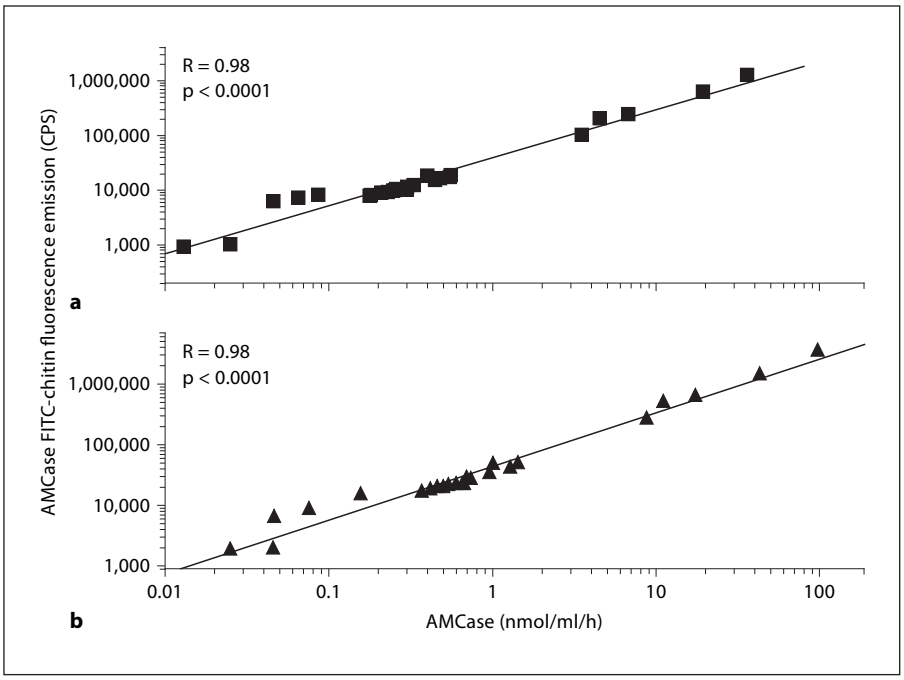

Fig. 1. Correlation between AMCase nmol/ml/h and FITC-chitin fluorescence emission (CPS). **a** Crude values. **b** Values normalized for protein content.

fluorescence (CPS) was recorded under the same assay conditions. A blank for control, composed of reagents (Tris-HCl 0.5 M + FITC-chitin), was used in each measurement. The interassay coefficient of variation (reproducibility) was <5%.

Inibition Test with Allosamidine. To confirm that the hydrolytic activity was due to AMCase, the dosage by both methods was repeated after neutralization of gastric juice chitinase activity with 9 μM of allosamidine (kindly provided by Dr. S. Sakuda), for 90 min at 37°C in a shaking water bath (160 rpm). This concentration is likely to completely inhibit chitinase activity [29]. The neutralization reaction was stopped by adding 180 μl of sodium dodecylsulfate (SDS) 10% wt/vol.

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. [27] and Tikhonov [28] methods.

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.

Statistical Analysis

The data are the mean of three determinations and the graphic representations were obtained by using the Prisma software. Median and range were calculated for each group.

Results

Among 25 studied patients, 4 who at physical and clinical examination were otherwise healthy, resulted negative for gastritis (patients 1, 2, 4, 10) and they represented the healthy controls. Only 13/25 (patients 5, 6, 12, 13, 15, 17–20, 22–25) showed signs of reflux esophagitis associated with chronic gastritis. Eight patients (3, 7–9, 11, 14, 16, 21) showed antral gastritis, gastric micropolyposis and mucosal gastric hypertrophy, and 9 patients (patients 11, 13, 16–18, 20, 21, 24, 25) resulted positive at *H. pylori* urease test and at ^{13}C-urea breath test.

Table 1 reports the chitinase activity of each gastric sample by the two different assays. The calculated activity is reported in nmol/ml/h of 4-methylumbelliferyl-beta-D-N,N′-diacetylchitobiose hydrolyzed and the FICT-chitin activity was reported as intensity of fluorescence (CPS). The consistency of the two methods is compared in figure 1, using a log scale, for crude values and for values normalized for protein content. For the two control samples, where no gastric juice was added, the level of activity was <0.2 nmol/ml/h and <10,000 fluorescence emission CPS, respectively. The standard samples added in each determination confirmed the reproducibility of the method with a variation coefficent <5% .

Gastric juices of 5/25 patients (2, 3, 4, 11 and 19) had very high chitinase activity (>3 nmol/ml/h). Five patients (6–10)

Table 1. Chitinolytic activity (AMCase) at pH 2 and FITC-chitin activity expressed in nmol/ml/h of hydrolyzed substrate and in fluorescence emission (CPS)

Subject No.	Sex	Age years	AMCase nmol/ml/h	AMCase, FITC-chitin fluorescence emission, CPS	Protein content (Lowry method) mg/ml	Gastroscopic diagnosis
1	M	73	0.4	18,362	335	no affection
2	F	53	3.54	102,234	340	no affection
3	F	74	6.8	242,315	333	chronic antral gastritis and gastric micropolyposis
4	F	58	36.27	1,254,782	315	no affection
5	F	59	0.26	10,425	316	chronic antral gastritis and suspected short Barrett esophagitis
6	M	64	0.086	8,125	465	reflux disease
7	M	43	0.025	1,013	466	chronic antral gastritis and duodenitis
8	M	45	0.065	7,231	724	chronic gastritis
9	M	40	0.013	916	438	antral gastritis
10	F	50	0.046	6,131	843	no affection
11	F	65	4.520	201,650	344	antral gastritis *H. pylori* urease test positive
12	F	39	0.178	7,900	325	antral gastritis, reflux disease
13	M	74	0.256	10,021	401	antral gastritis, reflux disease, *H. pylori* urease test positive
14	F	60	0.446	15,211	394	slight antral gastritis
15*	F	52	0.330	12,211	381	antral gastritis and leiomioma, hiatal hernia
16	F	53	0.303	11,402	421	antral gastritis, reflux disease, *H. pylori* urease test positive
17**	M	50	0.299	10,225	419	antral gastritis, reflux disease, *H. pylori* urease test positive
18	M	51	0.558	18,831	329	antral gastritis, reflux disease, *H. pylori* urease test positive
19	M	62	19.410	625,022	381	diffuse gastritis, reflux disease
20	M	48	0.249	9,821	422	antral gastritis, reflux disease, *H. pylori* urease test positive
21	M	45	0.480	16,428	621	antral gastritis, reflux disease, *H. pylori* urease test positive
22	F	54	0.549	17,241	361	antral gastritis, reflux disease, gastric micropolyposis
23	M	46	0.181	8,250	414	antral gastritis, reflux disease
24	M	40	0.230	9,201	388	antral gastritis, reflux disease, *H. pylori* urease test positive
25	M	60	0.210	8,881	421	diffuse gastritis, reflux disease, *H. pylori* urease test positive

* Intolerance to crustaceans. ** Patient from the Philippines.

Table 2. Chitinolytic activity (AMCase) at pH 2 and FITC-chitin activity expressed in nmol/ml/h of hydrolyzed subtracts and in fluorescence emission (CPS)

Patients	Chitinolytic activity (AMCase) nmol/ml/h	FITC-chitin activity expressed in fluorescence emission (CPS)	Age years	Sex M/F
5	0.046 (0.013–0.086)	6,131 (916–8,125)	45 (43–64)	4/1
15	0.299 (0.178–0.558)	10,425 (7,900–18,831)	52 (39–74)	9/6
5	6.80 (3.54–36.27)	242,315 (102,234–1,254,780)	62 (53–74)	1/4

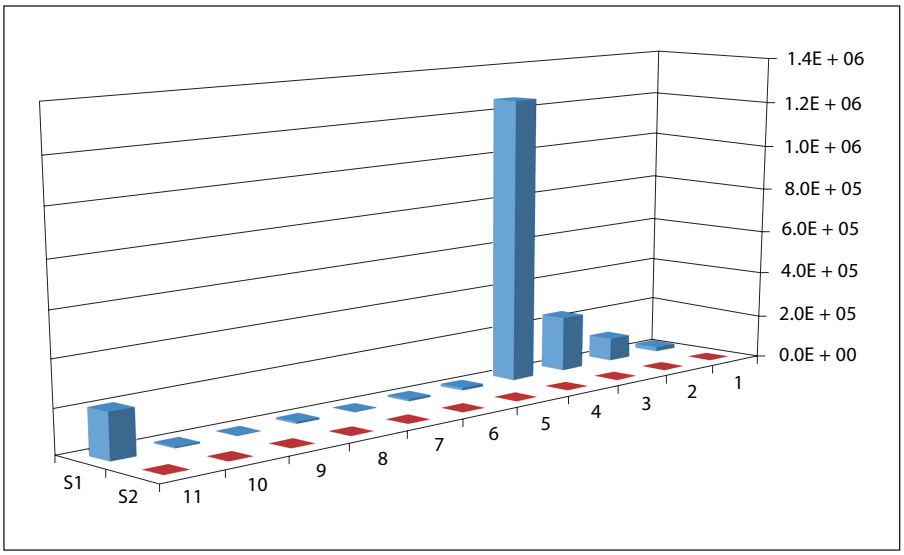

Fig. 2. FITC-chitin activity expressed in fluorescence emission (CPS) before (S1) and after (S2) inhibition with allosamidine (9 μM), in the first 11 subjects.

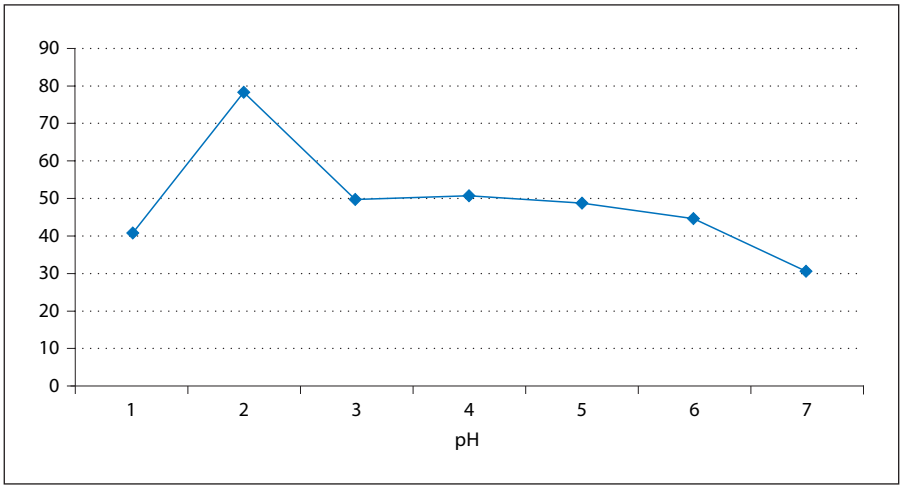

Fig. 3. AMCase activity (nmol/ml/h) of gastric juice of subject 4 at different pH values.

showed undosable AMCase activity (<0.20 nmol/ml/h) in both assay methods. In the remaining 15 samples (patients 1, 5, 12–18, 20–25) the AMCase activity was measurable (>0.20 <3 nmol/ml/h). The prevalence of *H. pylori* was uniformly distributed in the three groups examined and the patient from the Philippines showed undosable levels of chitinase activity. The patients were assigned to one of three groups according to the AMCase activity values (table 2). No statistically significant correlation was found between AMCase activity, sex and age of the patients.

In 11 samples after neutralization of chitinase activity with allosamidine, FITC-chitin emission fluorescence disappeared in all samples (fig. 2), confirming that the results are due specifically to the chitinolytic activity of gastric juice (table 1).

Measurement of the protein content obtained with the Lowry method [30] in gastric juices demonstrated no correlation between the protein content of gastric juice and the chitinase activity showing that the variation or absence of chitinase activity was not due to a dilution factor, but to a reduced AMCase production by the gastric cells.

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 (fig. 3), similarly to mouse AMCase.

Discussion

AMCase activity was present in the gastric juices of 20/25 Italian patients at different levels of activity from 0.178 to 36.27 nmol/ml/h and from 7,900 to 1,254,782 fluorescence emission CPS, according the used methods. The two methods are comparable, but the fluorescent emission CPS measurement, generated by FITC-chitin digestion, is more sensible in the same experimental conditions, supporting the advantage of this new natural substrate in the measurement of chitinase activity. This assay has advantages over the Boot et al. [27] method, since it is more sensitive and able to reveal low activity of chitinase, but is more complicated in the preparation of substrate. Another important observation emerging from the use of FITC-chitin is that in humans the gastric chitinase functions as an endochitinase differently from the other chitinases present in nature [31]. In fact, when we varied the incubation time from 30 to 120 min, 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 [32, 33], is characterized by a slow increment of fluorescence since the hydrolysis takes place at the terminal of chitin molecules.

Tikhonov et al. [28] in their experiments used a glucosaminidase to show that this enzyme does not generate fluorescence and only cleaves off single sugars, whereas human chitinases can cleave off larger fragments.

Boot et al. [27] reported in mouse bronchial epithelium and macrophages that only one chitinase is expressed (AMCase in bronchial tissue and Chit in macrophages). The difference is probably related to the specific function of these two chitinases which have their optimum activity in relation to the pH of the tissue where they are present. Chit should not be expressed in the stomach, at least under normal conditions. We demonstrated that Chit activity of plasma from Gaucher subjects (>800 nmol/ml/h) disappeared when the pH of the reaction medium was lowered from 5.2 to 1.5 with phosphoric acid (data not shown).

However, we cannot exclude that the macrophage cells present in gastric juice 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 this study, the curve indicating the rate of activity at different pH values was continuous and not bimodal as expected in the presence of two chitinases (AMCase and Chit). Moreover, no correlation was observed between the levels of chitinase activity and the gastroscopic aspect of gastric mucosa and 2 subjects (patients 2, 4) with elevated level of chitinase activity showed normal gastroscopy. On the contrary, paradoxically, the patients where *H. pylori* was detected by a urea test showed lower chitinase levels than the other patients who had evidence of gastritis based on gastroscopy.

We exclude that gastric chitinase activity in our patients could be associated with food residues since the patients did not consume food for at least 12 h 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 [32, 33].

The observation that 15/25 individuals (60%) did not show AMCase or FITC-chitin in gastric juices was surprising.

A confirmation that the absence of chitinase activity in gastric juices is not due to an artefact of unknown origin could be deduced from the identification of Caucasian individuals with no active chitinases in their plasma on a genetic background [29, 34]. In fact, 5–6% of healthy Caucasian individuals are deficient in Chit due to homozygous duplication of 24 bp in exon 10 of the CHIT gene and 35–45% of them are heterozygotes [34]. The consequence of this is a significant difference in the plasma Chit levels in healthy subjects from Africa, Sardinia and Sicily that depends on the allelic dosage in these individuals [19, 35]. Thus, Chit production, more abundant in African subjects of poor socioeconomic conditions, may reflect the genetics of individuals who maintain the wild-type CHIT gene (99%), while in Western countries the wild-type CHIT gene seems to have become redundant (55–65%), because of the higher socio-economic status of the latter [34].

If we consider that in the Sub-Saharan area the CHIT wild gene is conserved, whereas in Caucasian populations an inactive polymorphism is present [34], the disappearance of food containing chitin (adult insects and/or their larvae) could have favored the disappearance of AMCase activity in the gastric juice of humans as well as of Chit activity in plasma. Even if the potential to digest chitin has been documented in 20/25 (= 80%) of our Italian patients, we do not know the quantity of chitinases needed to effectively digest the chitin associated with insects and crustaceans. However the observation that the 5 individuals with not-dosable AMCase activity are in Weinberg equilibrium with the other 15 where the activity was measurable and the 5 individuals with very high AMCase activity suggest the possibility that also for the

AMCase a molecular mechanism similar to that demonstrated for Chit is active in the stomach. A study of AMCase polymorphism could clarify this important point confirming the role of environmental factors in the digestive function of humans [36].

The higher AMCase activity in tropical human populations with a higher rate of entomophagy could represent an adactative response to alimentary habits, conferring increased resistance against parasitic infection in these areas.

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