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Original Research

Absence of Allergic Reactions to Egg White Lysozyme Additive in Grana Padano Cheese

Amerigo Iaconelli, MD, Lucia Fiorentini, PhD, Sara Bruschi, PhD, Filippo Rossi, PhD, Geltrude Mingrone, MD, PhD, FACN, Gianfranco Piva, PhD

Dipartimento di Medicina Interna, Scienze specialistiche e dermatologia – Sez. Malattie del ricambio, Policlinico Agostino Gemelli, Università Cattolica del Sacro Cuore, Rome (A.I., G.M.), Istituto di Scienze degli Alimenti e della Nutrizione, Facoltà di Agraria, Università Cattolica del Sacro Cuore, Piacenza (L.F., S.B., F.R., G.P.), ITALY

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Objective: The negative effects on cheese quality of milk contaminated by spores of *Clostridium butyricum* and *Cl. tyrobutyricum* is prevented by the use of egg white lysozyme as additive. Since the presence of lysozyme in cheese could be possibly risky in allergic subjects, we aimed at investigating its absorption as well as serum IgE antibody titers after ingestion of Grana Padano cheese, an Italian DPO, long-ripened hard cheese, in white egg allergic subjects.

Methods: Cheese lysozyme was measured by HPLC. Ten healthy volunteers and 20 patients with hen egg hypersensitivity, RAST positive (binding $\geq 3\%$) to lysozyme and/or ovomucoid and ovalbumin received 15, 30 and 60 g of cheese at distance of at least 2 weeks each. Serum lysozyme was measured by ELISA and specific IgE binding to lysozyme by the radioallergosorbent test (RAST).

Results: The concentration of lysozyme in cheese was 155 ± 5 mg/kg. The area-under-the-curve of serum lysozyme after 15 g of cheese was 244.5 ± 14.0 in controls and 330.2 ± 9.9 in patients ($p < 0.01$). Similar results were obtained with 30 and 60 g of cheese. Only 3 patients (15%) showed positive IgE antibody responses to cheese (overall RAST mean $4.45 \pm 1.25\%$ vs. $4.24 \pm 1.02\%$ baseline, $p = ns$).

Conclusions: The amount of lysozyme absorbed with cheese was globally very low, although it was significantly lower in healthy controls than in allergic patients, where it induced an increase of IgE RAST score in 15% of subjects, without any clinical reaction. Therefore, the use of lysozyme as additive in Grana Padano cheese, does not appear to be harmful in egg allergic subjects.

INTRODUCTION

The prevalence of food allergies in childhood varies from 6% to 8% in the general population. Recently, using food-specific IgE tests, Bohmbal et al [1] showed that 5% of a consecutive series of 242 pediatric otolaryngology patients were allergic to egg white. A cross-sectional epidemiological study [2] on 1141 randomly selected young adults, aged from 20 to 45 years, revealed that 1.3% had probable IgE-mediated food allergy, of which 0.09% was for egg.

With the advent of improved immunological assays such as the radioallergosorbent test (RAST) the ability to define which

proteins are involved in IgE-mediated egg allergy is greatly improved. Hoffman et al [3] demonstrated that ovalbumin, ovomucoid and ovotransferrin were important allergens, whereas lysozyme was only a weak allergen. In contrast, Holen and Elsayed [4] found that lysozyme bound strongly to IgE in all of the sera of egg-allergic individuals that they studied, and concluded that lysozyme was one of the major allergens of egg white.

Contamination of cheese milk with spores of acetate-fermenting clostridia, in particular *Clostridium tyrobutyricum*, originates mainly from the use of silage in the feeding of dairy cattle. Since these spores easily survive heating temperature, as

Address correspondence to: Dr. Geltrude Mingrone, Istituto di Medicina Interna e Geriatria, Policlinico Agostino Gemelli, Largo Agostino Gemelli 8, 00168 Roma, ITALY.
E-mail: gmingrone@rm.unicatt.it

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Abbreviations: DPO = protected designation of origin, RAST = Radio-allergosorbent test.

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that utilized in the production of Grana Padano (56°C), they provoke a typical defect called "late gas" [5] or "late blowing" [6] during the ripening of some cheese, like Grana Padano.

Egg white lysozyme has been shown to prevent the late gas defect of Edam cheese (Edam is a typical Dutch village, world famous for its cheeses) completely even if spores derived from lysozyme-resistant vegetative cells have been used to contaminate the cheese milk [5], lysozyme can also suppress the growth of *Clostridia* in Grana Padano [6].

Germination of *Clostridium tyrobutyricum* [7] as well as *L. monocytogenes* Scott A [8] spores into vegetative cells is sensitive to lysozyme. Therefore, lysozyme from white egg was used to protect cheese against decomposing bacteria and to prevent late blowing in hard ripened cheese.

Grana Padano is an Italian DPO (protected designation of origin), long-ripened hard cheese produced with raw milk and its manufacturers have started to employ lysozyme extracted from egg white to counter late blowing caused by the outgrowth of clostridial spores (*Cl. butyricum* and *Cl. tyrobutyricum*) present in raw milk [9–11], for at least 20 years [12]. The hypothesis of the present investigation was that the presence of lysozyme in Grana Padano might be risky in subjects with white egg allergy, possibly inducing allergic reactions. To this end we aimed at investigating lysozyme absorption as an intact molecule in healthy and white egg allergic subjects as well as their serum IgE antibody titers after increasing ingested amounts, from 15 to 60 g of this cheese.

MATERIALS AND METHODS

Analysis of Lysozyme in the Grana Padano Cheese

Lysozyme content in samples of Grana Padano cheese, produced in July 2004, with the addition of 2,5 g of lysozima in 100 kg of milk (Lysozima trade products Hansozyma by CHR HANSEN, Hørsholm, Denmark, cod. 677163 lot n° H1044), after 18 months of ripening, was quantified by HPLC method described by ISO TC 34/SC 5N [13]. Casein and denaturated whey proteins from milk and milk products were precipitated isoelectrically at pH 4.3 (cheese and solid milk products) or pH 2.2 (milk and liquid milk products). Acid-soluble lysozyme was then determined by reversed-phase HPLC and fluorescence detection (Fig. 1).

The method resulted suitable for measuring low levels of lysozyme with a quantification limit of 0.5 mg per kg.

Extraction of Lysozyme from Cheese. Two gram samples of Grana Padano cheese were weighed into a 100 ml beaker and added with 20 ml of sodium chloride solution 1mol/L. The pH of the obtained solution was adjusted drop wise sodium hydroxide solution 0.1 mol/L to pH 6.0. The test portion was homogenized with Ultra Turrax (T 25 basic IKA WERKE Ghiaroni, Bucinasco MI, Italy) at medium speed for 30 s. The homogenizer was rinsed with 10 ml of sodium chloride solution

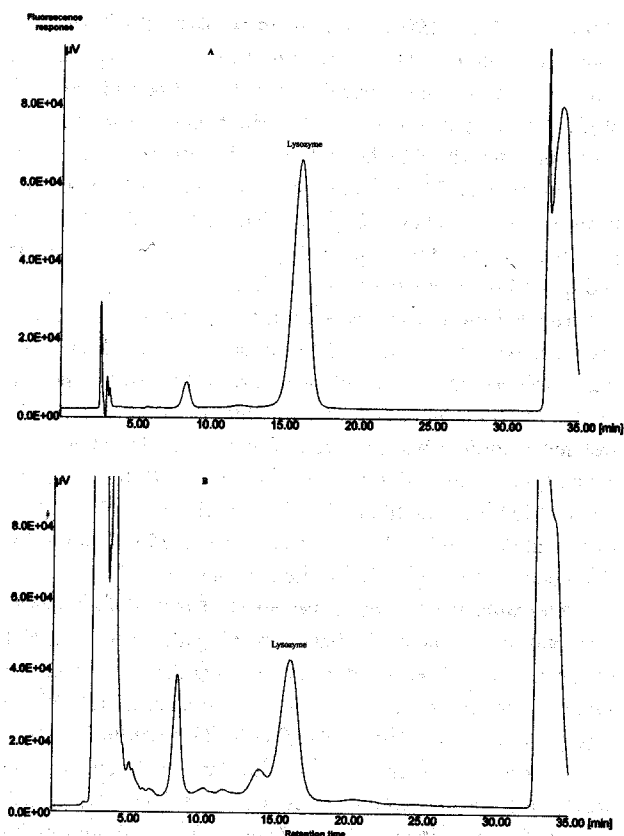


Fig. 1. A) HPLC of extract of cheese containing lysozyme (arrow). B) HPLC of solution standard lysozyme (arrow).

1 mol/L. The rising was added to the test solution. The beaker, containing the test solution, was stirred by a magnetic stirrer at room temperature for 1 h.

The pH of the test portion obtained from cheese was adjusted to pH 4.3 by using hydrochloric acid 0.1 mol/L. The test solution was transferred into a 50 ml one-mark volumetric flask. Sodium chloride solution 1 mol/L was used to rinse the 100 ml beaker and to dilute to the 50 ml mark and then the solution was mixed. The test solution was allowed to stand at room temperature for 15 min.

Firstly, the test solution was filtered through a fluted filter (Schleicher & Schuell 595 12 n° 10311647, MicroScience, Dassel Germany) and then through the membrane filter (Holder 13 mm sst Swinney Siringe, filter type 0.22 μ m GSWPO1300 13 mm Millipore Bedford, MA, USA) directly into a HPLC vial.

HPLC Conditions. The following conditions were used. Column type: reversed-phase polymeric column (PLRP-S 250 \times 4.6mm, 300 Å pore size, 5 μ m from Lab service analitica BO, Italy). Eluting solvents: solution I (1 ml trifluoroacetic acid in 1 L water), solution II (1 ml trifluoroacetic acid in 1L acetonitrile); solvent A stock solution I: stock solution II = 100: 390 (w/w) solvent B stock solution II. Eluting conditions expressed as proportion of solvent A: 0– 18min,

100%; 18–24min, 100–50%; 24–25min, 50%; 25–26min, 50–100%; 26–35min, 100%. Flow rate 1 mL min^{-1} . Run to run time: 35 min. Column temperature 45°C . Injected volume: $50\mu\text{L}$. HPLC apparatus (Jasco AS-950 Tokyo, Japan): fluorescence detector (F9-920 Jasco Tokyo, Japan); fluorescence at 280 nm ex. and 340 nm em were recorded and reprocessed using software packages Borwing. The area of the lysozyme peak was measured using the valley to valley integration mode and quantified by a single point calibration.

Preparation of Lysozyme Standard Solution. Ten milligrams of lysozyme (cod L-6876 Sigma, St. Louis, MO USA) were weighed into a 10 ml one-mark volumetric flask and diluted to 10 ml mark with sodium chloride solution 1 mol/L. Reference portions were prepared fresh daily. Eighty microliters of the lysozyme solution were pipetted into a 10 ml one-mark volumetric flask and diluted to 10 ml mark with sodium chloride solution 1 mol/L. The so obtained standard working solution contains 8.0 mg of lysozyme per liter.

Determination of the Total Solids Content of Cheese. The total solid content, the mass remaining after completion of the heating was determined by evaporating the water from the sample in the presence of sand at a temperature of 102°C in a drying oven (WTB Binder, 19115300002000, Tuttlingen, Germany) according to International standard [14]. The sample was grated and the ground mass was mixed quickly. A dish containing approximately 25 g of sand was heated with its lid and stirring rod in the drying oven at 102°C for 1h. The dish was transferred with the lid and rod on top to a desiccator, was allowed to cool for at least 45 min and weighed. The sand was tilted to one side of the dish, about 3.0 g of the prepared sample was placed on a clear space, the lid with the stirring rod on top was replaced and then the dish was weighed. The test portion was mixed with the sand, and the mixture was spread evenly over the bottom of the dish, the stirring end of the rod was left in the mixture. The dish was heated in the drying oven at 102°C for 3 h, then was placed with lid and stirring rod in the desiccator to cool and was weighed.

Subjects

Ten adult healthy volunteers, aged 20–50 years (27 ± 9), with a BMI of $22.8 \pm 1.8\text{ kg/m}^2$, were enrolled in the study.

Twenty age and sex-matched subjects (24 ± 8 years and BMI $22.8 \pm 1.7\text{ kg/m}^2$) with white egg allergy were also enrolled. Eleven of them were RAST positive (binding $\geq 3\%$) to both lysozyme and ovomucoid (28 kDa), 6 were positive to both lysozyme and ovalbumin (43 kDa), and 3 were positive only to ovomucoid.

Allergic patients were hospitalized for 48 hours. The patients who participated in the present study had never had any experiences of gastrointestinal and respiratory symptoms on exposure with hen-egg in their life in view of life-threatening risk for patients.

Oral Challenge Methodology

Oral challenges were carried out according to the procedure including placebo, food and single-blind tests [15], with the informed consent of the patients. The protocol was approved by the Ethical Committee of the Università Cattolica, School of Medicine in Rome, Italy. Mashed potatoes were used as placebo since they are not generally allergenic. The cheese was grated in the vehicle.

The time interval between the three progressions (15, 30 and 60 g) was 2 week or more.

Monitoring of Oral Challenges

Pulse frequency, blood pressure (BP), breath sounds, peak flow rate (PFR) and any skin and/or mucose color modifications were monitored every 20 min, during 12 h. The clinical score for atopic dermatitis (SCORAD) was calculated 8 and at 24 h later [16].

Objective clinical signs were taken into account: tachycardia, drop in BP (=3 points) urticaria, angio-oedema, audible wheeze, cough, drop in PFR, rhinorrhoea, conjunctival redness, diarrhoea, vomiting, immediate rash on eczema and delayed exacerbation of eczema. A SCORAD > 10 points was taken as positive criteria of allergic reaction do cheese.

After leaving hospital the patients were instructed to go immediately to the nearest hospital (average time required 30 min), if symptoms, such as acute urticaria, should develop.

Blood was collected before administration and 15, 30, 60, 90 and 120 min after cheese ingestion. Blood was left at room temperature for 30 min, then the serum was separated by centrifugation at 1600 g for 10 min. The serum was kept frozen at -20°C until assay.

Lysozyme Assay in Sera

Lysozyme concentration in serum was measured by Lysozyme EIA Kit, a sandwich ELISA assay, using two highly specific lysozyme antibodies (Diagnostic Instruments GmbH, Morburg, Germany). The sensitivity of the assay was 0.78–50 ng/ml.

Lysozyme HPLC Purification

Lysozyme from Sigma Aldrich (98% purity) was further purified by reversed-phase HPLC on a HP C_{18} column (4.6 by 250 mm) (Alltech Associates, Inc. Deerfield, IL, USA) with a linear gradient of acetonitrile that contained 0.13% heptafluorobutyric acid as the ion-pairing agent [17].

Radioallergosorbent Tests (RAST)

Filter paper discs (6 mm) were activated with cyanogens bromide by the methods of Ceska et al [18]. The optimal amount of each protein to be coupled was determined by constructing a binding curve in which increasing amounts of

the lysozyme were added to CNBr-activated paper [19]. After incubation of the RAST disk in 50 μ l of the patient's serum for 18 h at 4°C, the RAST disk was washed three times in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-Tween). The disk was placed in 50 μ l of 125 I-conjugated anti-human IgE (Dainabot Co., Tokyo, Japan) for 18 h at room temperature and rinsed with PBS-Tween three times, followed by counting in a gamma counter. Per-cent binding was calculated by the following formula: (bound isotope count / added total count isotope of 125 I-antihuman IgE) \times 100.

Statistics

Data are reported as mean \pm SEM, unless otherwise specified. Data analyses were performed with PROC ANOVA of SAS 8.0 statistical package. Two-sided $P < 0.05$ was regarded as significant.

The Wilcoxon signed rank test was performed to compare data from the same subjects before and after cheese oral test, the P values were adjusted using the Bonferroni method. The comparison between groups was performed by Mann-Whitney method for non-parametric independent two-group comparisons. The distribution of the residuals, testing for normality and checking the linearity assumptions in the model by means of standard scatter plots. The areas under the curve of the lysozyme time courses were calculated using a trapezoidal rule.

RESULTS

Lysozyme Concentration in Cheese

Grana Padano cheese used for the test, was produced in July 2004 after 18 months of ripening. Egg lysozyme (trade products Hansozyma) was used in cheese-making to prevent late blowing by hindering growth of *Clostridium tyrobutyricum*.

The concentration of lysozyme found in sample of Grana Padano cheese was 155 ± 5 mg/kg and the dry matter expressed in per cent by weight was 67.90.

Plasma Lysozyme Levels

The area under the curve (AUC) of the serum lysozyme concentrations was significantly higher in patients than in controls independently of the amounts of Grana Padano challenged (Table 1).

No dose-response curve was detected using progressively higher amounts of Grana Padano.

Serum Lysozyme Specific IgE

None of the patients developed positive reactions either immediate - i.e. developed within 1 hour after consuming the cheese-, late - i.e. those symptoms which occurred within several to 24 hours -, or delayed - i.e. those symptoms that occurred after several days.

Table 1. AUC (Area under the Curve, μ g/ml \cdot min, Mean \pm SE) of Lysozyme Serum Concentration, after Consumption of Increasing amount of Grana Padano Cheese, in Egg White Sensitive and Non-Sensitive Subjects

Amount of administered Grana Padano cheese (g)	AUC (μ g/ml \cdot min)	
	Sensitive subjects	Non Sensitive subjects
5	330.2 \pm 9.9 ^b	244.5 \pm 14.0 ^a
15	330.9 \pm 10.1 ^b	235.8 \pm 14.2 ^a
60	335.5 \pm 10.3 ^b	254.4 \pm 14.5 ^a

^{a,b} ($p < 0.01$)

RAST values were expressed as the mean percent bound of the total 125 I-labelled anti-IgE added. Positive RAST was defined as the percentage bound greater than 3%. Since no significant differences in serum lysozyme levels, no clinical reactions and no significant differences among values in the different experimental sessions were observed, the RAST values reported in the results were averaged from the data relative to the three cheese challenges.

RAST values in controls were $0.84 \pm 0.47\%$.

Among the 20 patients with a history of egg white allergy tested, 17 (85%) had baseline serum lysozyme specific IgE (mean \pm SD = $4.24 \pm 1.02\%$), and between the latter only 3 (15%) showed positive IgE antibody responses to Grana Padano cheese (subject 5 from 4.7% to 5.10%, subject 7 from 5.00 to 6.10% and subject 18 from 4.33 to 5.63% (overall RAST mean $4.45 \pm 1.25\%$, $P = n.s.$).

DISCUSSION

Hen egg white lysozyme is a 14.3 kDa protein formed by a single chain of 129 amino acids. Its structure consists of 5-7 α helices and a 3-stranded antiparallel β sheet [20]. Its function is to hydrolyze the β (1-4) glycosidic bond between residues of *N*-acetylmuramic acid and *N*-acetylglucosamine in certain polysaccharides contained in many bacterial cell walls.

For these antibacterial properties, lysozyme from hen egg white was used in cheese-making to prevent late blowing by hindering growth of *Clostridium tyrobutyricum*. In 1992 the FAO/WHO Expert Committee on Food Additives approved the utilization of lysozyme as a cheese additive with a tolerability of 10-35 g/100 L of milk used in cheese production, corresponding to 10-35 g/100 kg of finished product [21]. Indeed, the average concentration of lysozyme we have found in samples of Grana Padano cheese was 155 mg/kg, i.e. 15 g/100 kg. This value is, therefore, in the range allowed, and rather in the low limit.

The major goals of present study was to investigate the degree of lysozyme absorption in healthy controls and in age, sex and BMI matched subjects with clinical story of white egg allergy.

The plasma lysozyme titers were almost unmodified after ingestion of the Grana Padano cheese and no dose-response curve was detected in either group of subjects. However, in allergic subjects the AUC was slightly but significantly higher for all the amounts of cheese ingested. These results suggest that subjects allergic to white egg proteins might have a higher intestinal permeability to proteins than controls.

The intestinal epithelium of healthy adults represents a barrier towards food proteins, to which it is generally considered to be virtually impermeable. Furthermore, the activity of digestive enzymes allows an extensive degradation of food proteins, thus contributing to restrict the entry of intact, undigested proteins into the body [22]. However, some studies suggest that a small, but significant, amount of biologically and/or antigenically active peptides and proteins passes through the intestinal epithelium [23–25].

Recently Nishikawa et al [26] reported that cationization of lysozyme increased its intestinal absorption likely through the increase in the isoelectrical point of the enzyme. When lysozyme is charged positively it can interact electrostatically with the negatively charged proteoglycans of the intestinal cell surface.

Indeed, increased intestinal permeability has been ascribed as a possible cause of food allergy in adults [27].

The other main finding of this study is that no allergic reactions were provoked after oral tests with Grana Padano cheese in subjects allergic to white egg proteins at doses of 0.8, 2.3 and 9.3 mg of lysozyme content in 5, 15 and 60 g of this cheese, respectively.

Lysozyme has been identified as an important allergen in hen egg white. IgE-mediated reactions (type I), which are characterized by the rapid appearance of the allergic reaction or anaphylaxis after the ingestion of the offending foods, afflict about 1–2% of adults and 5–8% of infants [28,29].

Hen eggs, and in particular egg white proteins, represent one of the most frequent causes of adverse reactions to food [30].

The most represented proteins in egg white are ovalbumin accounting for 54% of the whole protein content; ovomucoid, 11%, ovotransferrin, 12%, ovomucin, 3.5%, and lysozyme, 3.5% [31].

Finally to our knowledge, there is only one study (Kanny FS et al) dealing with the prevalence of lysozyme sensitization in a clinically allergic to egg population of 52 subjects, ranging from 6 months to 45 years. The Authors [31] report that 18 out of 52 subjects, i.e. 35% of their population, had anti-lysozyme IgE.

Pichler and Campi [32] described local or systemic allergic reactions in 7 patients who received a lysozyme, nystatin, and tetracycline containing vaginal suppository because of suspected vaginal infection. Five of these seven subjects had positive skin tests to ovomucoid and lysozyme, but in none of them lysozyme-specific IgE were found in the circulatory stream. It is, however, noteworthy that although four of the

above patients had urticaria or anaphylaxis after treatment for at least three days, none of them developed egg allergy. A possible explanation is that lysozyme was not completely pure, but it could contain other allergens as contaminants.

To summarize, no allergic reaction was induced by Grana Padano cheese. The amount of lysozyme absorbed after ingestion of 15, 30 or 60 g of Grana Padano cheese was globally very low, although it was significantly higher in allergic patients than in healthy controls. In spite of the low degree of absorption as an intact molecule, lysozyme, content in the cheese, increased the IgE RAST score in egg white allergic subjects.

In conclusion, the use of lysozyme as an additive in cheese, at the recommended doses for the production of Grana Padano production, does not appear to be harmful in egg allergic subjects.

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