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Key words: lysozyme, cheese, allergenicity

Objective: Lysozyme is an enzyme that hydrolyzes bacterial peptidoglycans. For this reason, it is used in cheese manufacturing in order to prevent a defect of long-ripened hard cheese called "late blowing" due to the outgrowth of spores of *Clostridium tyrobutyricum* and *Clostridium butyricum*. Moreover, germination of *Listeria monocytogenes* spores into vegetative cells is also sensitive to lysozyme. The enzyme can be an allergenic molecule, and for this reason there are concerns about its use in food industry. The immunological and clinical response of consumption of lysozyme-containing cheese has been evaluated in 25 egg-sensitive subjects with or without lysozyme sensitization.

Methods: A total of 25 egg-sensitive subjects were enrolled in this study. All the subjects were already treated for egg-sensitization and presented a positive skin prick test. All the subjects had a body mass index ≤ 25 kg/m² and were in the age range of 20–50 years. Each subject was studied twice and received randomly 30 g of Grana Padano (containing lysozyme) or TrentinGrana cheese (lysozyme-free) of two different aging periods: 16 or 24 months. A washout period of 1 week between each cheese intake was adopted. Blood samples were taken in fasting conditions and 1 hour after cheese intake and IgA, total IgE, and lysozyme-, ovomucoid-, and ovalbumin-specific IgE were measured.

Results: No adverse reactions were observed in both groups of patients after cheese samples were given. Lysozyme did not determine any variation of specific IgE compared with basal level. In lysozyme-sensitive patients a significant relationship between IgA and lysozyme-specific IgE was observed when lysozyme-containing cheese was given, confirming that lysozyme can pass the gut barrier.

Conclusions: Neither adverse events nor immunological responses were observed after ingestion of cheese containing lysozyme. However, the immunological properties of peptides deriving from cheese protein hydrolysis need to be clarified, as does the effect of lysozyme on bacterial proteolytic activity.

INTRODUCTION

Lysozyme is a 14.3 kDa enzyme, known as muramidase or N-acetylmuramide glycanhydrolase (EC. 3.2.1.17), made up of a single-strain chain of 129 amino acids consisting in 5–7 α helices and 3-stranded antiparallel β sheets. The enzyme works

by attacking peptidoglycans of bacteria cell walls and hydrolyzing the glycosidic bond (1–4) that connects N-acetylmuramic acid with N-acetylglucosamine [1]. For these antibacterial properties, lysozyme was used in order to prevent a typical defect of long-ripened hard cheese called "late blowing," which is due to the outgrowth of spores of *Clostridium*

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Table 1. Chemical Composition of Tested Cheese

Sample	Lysozyme (mg/kg)	Aging (mo)	Moisture (g/100 g)	Protein (g/100 g)	Lipids (g/100 g)
A	0	16	34.23	31.55	28.00
B	0	24	33.37	32.22	27.00
C	121.1	14.6	33.66	31.91	28.50
D	130.3	24.3	31.00	32.90	30.00

tyrobutyricum and *Clostridium butyricum* present in the raw milk [2]. Moreover, it was found that germination of *Listeria monocytogenes* spores into vegetative cells is sensitive to lysozyme too [3]. In Grana Padano, the lysozyme concentration was found to be around 155 mg/kg [4] (i.e., in the low range of the limits established by the European Union law, namely 100–350 mg/kg of finished product; European Parliament and Council Directive No. 95/2/EC). Lysozyme used for cheese production is obtained from egg white, which is classified among the foods responsible for 90% of food allergies. A cross-sectional epidemiological study on adults aged from 20 to 45 years, revealed that 1.3% had IgE-mediated food allergy, of which 0.09% was allergy to eggs [5]. However, the role of lysozyme as an allergen in eggs is still debated: Fremont et al. [6] showed that anti-egg lysozyme IgE antibodies were found in 35% of allergic egg consumers; Hoffman et al. [7] demonstrated that ovomucoid and ovotransferrin are the major egg allergens, whereas lysozyme is a weak one; Holen and Elsayed [8] observed that lysozyme bound strongly to IgE in all sera of egg-allergic individuals that they studied. In a previous work [4], we found that only 15% of subjects with hen-egg hypersensitivity who were fed 30 or 60 g of Grana Padano showed positive IgE response to cheese, but also that lysozyme levels in plasma were higher in the allergic subjects.

Indeed, some studies have already demonstrated that small amounts of biologically or antigenically active proteins/peptides can pass the intestinal barrier [9–16], and increased intestinal permeability has been implicated as a possible cause of food allergy in adults [17]. IgA-S (secretory IgA) plays an important role in protection of the mucosal barrier. IgA-S is a component of the gut-associated lymphoid tissue and provides noninflammatory first-line defense at the mucosal local level by showing an immune-exclusion activity that traps antigens [18]. There is evidence that a significant number of allergic individuals and subjects with celiac disease have associated IgA deficiency [19] and that IgA deficiency is linked to the development of gastrointestinal food hypersensitivity [17,20]. The levels of local IgA-S are related to serum IgA levels, instead higher IgA-S are related to lower IgE, that represent the second-line local defense, which leads to the release of histamine and inflammatory immune response [18].

The aim of the present study was to evaluate the immune-sensitizing activity of the peptides from milk in Grana Padano or TrentinGrana (hard-ripened cheese, free from lysozyme), by measuring the levels of IgA and IgE in sera of subjects fed with either type of cheese.

MATERIALS AND METHODS

Cheese Analysis

Cheese samples were characterized for their content in dry matter [21], crude protein [22], lipids [23], and ash [24]. Lysozyme was determined by high-performance liquid chromatography according to the method proposed by Pellegrino and Tirelli [25]. Biogenic amines were determined according to Smith and Davies [26].

The two types of cheese, manufactured with or without lysozyme (Fordrass SA, Lugano, Italy) and named, respectively, Grana Padano and TrentinGrana, were provided by the Grana Padano DOP Consortium. Cheesemaking took place in two dairy industries, and cheese samples were provided as servings of 30 g each. For each type of cheese two different ripening times were used: 16 and 24 months. The trial was carried out as a double-blind experiment.

Clinical Trials

The experiment was carried out in two clinical units, namely the Agostino Gemelli Hospital, Catholic University, in Rome (Department of Internal Medicine) and the “San Timoteo” Hospital, in Termoli, Campobasso (Allergology Unit, Hematology Department), the latter under the responsibility of the Catholic University in Campobasso. Fifteen subjects were enrolled in Rome and 10 subjects in Termoli. All subjects were already treated in the two centers for egg-sensitization and presented positive skin-prick test results.

Because egg sensitivity is due to several molecules (ovalbumin, ovomucoid, and lysozyme), the subjects were recruited according to their sensitivity to egg proteins. People

Table 2. Content in Biogenic Amines (mg/kg Fresh Weight) of Tested Cheese

Amines	Without Lysozyme		With Lysozyme	
	A	B	C	D
Putrescine	4.57	5.27	6.88	5.80
Cadaverine	0.60	1.82	3.69	4.14
Histamine	171.00	10.45	44.43	134.51
Tyramine	7.99	1.11	5.70	3.69
Spermidine	0.57	0.37	0.51	0.43
Spermine	5.22	6.75	3.80	4.19
Total	189.95	25.77	65.01	152.76

Table 3. Blood Basal Values (Mean $\pm$ SD) for IgA, Total IgE, and Ovalbumin-, Ovomucoid-, and Lysozyme-Specific IgE in Egg-Sensitive Subjects Recruited in Two Different Locations and Having Different Sensitivity to Lysozyme

Location	IgA (mg/ml)	Total IgE (ng/ml)	IgE Ovalbumin (kU/l)	IgE Ovomucoid (kU/l)	IgE Lysozyme (kU/l)
Termoli, Campobasso	3.93 $\pm$ 1.53	89.44 $\pm$ 53.75	21.36 $\pm$ 23.41	9.04 $\pm$ 12.78	0.11 $\pm$ 0.075
Roma	3.75 $\pm$ 1.43	84.26 $\pm$ 66.03	0.22 $\pm$ 0.086	16.38 $\pm$ 10.98	1.81 $\pm$ 1.71
<i>p</i>	0.7768	0.8443	0.0018	0.1342	0.0073

in Campobasso had a high ovalbumin sensitivity but showed low lysozyme-specific levels of IgE. The subjects involved in the trial carried out at the Gemelli Hospital were lysozyme-sensitive but had a low level of albumin-specific IgE.

All the subjects had a body mass index ≤ 25 kg/m² and were in the age range of 20–50 years. The protocol was approved by the Ethical Committee of Catholic University [Prot.cl.751 (A.1439)(C.F/2008)], and the subjects involved signed an informed consent.

Any subject involved in the experiment randomly received 30 g of Grana Padano (containing lysozyme) or TrentinGrana cheese (lysozyme-free) of two different aging periods (16 or 24 months) on two separate occasions. A washout period of 1 week between each cheese intake was adopted.

A blood sample (3 ml) was taken at fasting and 1 hour after cheese intake, as suggested by Liccardi et al. [27].

The samples were immediately centrifuged and divided equally into several tubes and stored at -80°C until analysis. IgA, total IgE, and lysozyme-, ovomucoid-, and ovalbumin-specific IgE were determined according to Fall et al. [28].

Statistical Analysis

The normality of data distribution was verified using the Shapiro-Wilk test. If data were not normally distributed, mean comparison was performed using the Mann-Whitney *U* test; otherwise, the Student *t* test was used. The comparison of observed IgE levels with the respective basal levels were analyzed using the Dunnett test.

Statistical analyses were done with the software SAS 9.2 (SAS Institute, Cary, NC) [29]. The correlation between IgE levels was carried out with the PROC CORR of SAS 9.2.

RESULTS

Cheese Composition

The chemical composition of cheese samples is reported in Table 1: Data fit well with the typical composition of these foods [30]. The estimated lysozyme intake was 36.4 mg and 39.0 mg for the samples aged 16 and 24 months, respectively.

Biogenic amines are important constituents of cheese that can elicit adverse reactions. Table 2 reports the levels of biogenic amines in the samples of tested cheese. In both cheese types histamine is the more abundant molecule, confirming

data of Naila et al. [31] and Rossi et al. [32]; however, lysozyme-containing cheese shows increasing levels of amines during aging, due to a sharp increase in histamine, whereas an opposite pattern can be observed in lysozyme-free cheese. This could be due to a change in proteolytic microflora induced by lysozyme as described by Neviani and Carini [33].

Clinical Results

No adverse reactions were observed in either group of patients after administration of cheese samples. Data for total and lysozyme-specific IgE were normally distributed, whereas concentrations of IgE specific for ovalbumin, ovomucoid, and IgA were not; for this reason data were analyzed using a nonparametric method.

Basal Levels of IgE. Table 3 reports data of IgA and IgE basal levels. As expected on the basis of different dietary habits, people recruited in the two centers had a different pattern of IgE.

Blood Concentration of IgA and IgE. Lysozyme did not determine any variation of specific IgE levels compared with their basal level, although a reduction in IgA and total IgE was observed (Table 4). In Table 5 data are expressed as a function of basal levels (equal to 100). With both groups of subjects, the consumption of 16-month aged, lysozyme-containing cheese did not affect IgA and IgE variations compared to lysozyme-free cheese. When the volunteers received the 24-month aged samples, a higher level of IgA and total Ig E was observed in both groups. In the group with the highest basal level of ovomucoid-specific IgE, the consumption of lysozyme-free cheese resulted in a higher concentration of specific IgE compared with lysozyme-containing cheese.

In both groups of subjects blood levels of total IgE increased with the increase in ovalbumin-specific IgE levels; on the other hand, ovalbumin- and ovomucoid-specific IgE were inversely related in ovalbumin-sensitive subjects but not in lysozyme-sensitive ones. This different relationship was not affected by the presence of lysozyme in the administered cheese (Table 6).

In lysozyme-sensitive people the levels of total IgE and ovomucoid-specific IgE were highly correlated (Table 6).

In lysozyme-sensitive patients a significant relationship between IgA and lysozyme-specific IgE levels was observed when lysozyme-containing cheese was given (Table 6).

Table 4. Variations, Compared with Basal Value, in Blood Concentration (mean $\pm$ SD) of IgA, total IgE and Ovalbumin-, Ovomucoid-, and Lysozyme-Specific IgE in Egg-Sensitive Subjects, Having Different Lysozyme Sensitivity and Receiving Cheese Aged 16 Or 24 Months and Produced with or without the Use of Lysozyme. Blood Samples Were Taken after 1 Hour after Cheese Consumption

Location: Termoli, Campobasso					
Immunoglobulin (Ig)	Basal Value T ₀	Immunoglobulin Levels after 1 h (T ₁)			
		Without Lysozyme		With Lysozyme	
		A (16 mo)	B (24 mo)	C (16 mo)	D (24 mo)
IgA (mg/ml)	3.93 $\pm$ 1.53	3.50 $\pm$ 1.58	3.62 $\pm$ 1.46	3.32 $\pm$ 1.45*	2.53 $\pm$ 1.29**
IgE total (ng/ml)	89.44 $\pm$ 53.75	87.64 $\pm$ 52.80	84.51 $\pm$ 54.25	85.02 $\pm$ 41.97	68.03 $\pm$ 36.35**
IgE ovalbumin (kU/l)	21.36 $\pm$ 23.41	21.22 $\pm$ 23.18	21.49 $\pm$ 23.40	21.22 $\pm$ 23.28	21.24 $\pm$ 23.19
IgE ovomucoid (kU/l)	9.04 $\pm$ 12.78	9.07 $\pm$ 12.92	8.99 $\pm$ 12.70	9.08 $\pm$ 12.95	9.04 $\pm$ 12.83
IgE lysozyme (kU/l)	0.111 $\pm$ 0.075	0.114 $\pm$ 0.075	0.112 $\pm$ 0.078	0.109 $\pm$ 0.070	0.110 $\pm$ 0.075

* $p < 0.05$; ** $p < 0.01$ compared with control.

Table 5. Variations, Compared with Basal Value = 100, in Blood Concentration (Mean $\pm$ SD) of IgA, Total IgE, and Ovalbumin-, Ovomucoid-, and Lysozyme-Specific IgE in Egg-Sensitive Subjects, Having Different Lysozyme Sensitivity and Receiving Cheese Aged 16 or 24 Months and Produced with or without the Use of Lysozyme

Immunoglobulin (Ig)	Location: Campobasso			Location: Rome		
	16 mo Aging		p	16 mo Aging		p
	With Lysozyme (C)	Without Lysozyme (A)		With Lysozyme (C)	Without Lysozyme (A)	
IgA (mg/ml) ^U	82.93 $\pm$ 11.89	87.04 $\pm$ 11.05	0.3099	90.73 $\pm$ 16.56	97.25 $\pm$ 7.71	0.2540
IgE total (ng/ml) [†]	100.32 $\pm$ 16.08	100.10 $\pm$ 8.97	0.9638	95.68 $\pm$ 14.24	98.64 $\pm$ 11.93	0.3741
IgE ovalbumin (kU/l) ^U	99.62 $\pm$ 2.41	99.65 $\pm$ 4.80	0.8946	97.68 $\pm$ 29.82	99.16 $\pm$ 18.08	0.5755
IgE ovomucoid (kU/l) ^U	99.78 $\pm$ 7.11	97.59 $\pm$ 9.44	0.8946	97.70 $\pm$ 7.23	99.98 $\pm$ 1.14	0.2717
IgE lysozyme (kU/l) [†]	100.07 $\pm$ 7.62	104.62 $\pm$ 4.90	0.1716	99.13 $\pm$ 3.32	101.54 $\pm$ 4.48	0.1499

[†] Means comparison performed with Student t test.

^U Means comparison with nonparametric U test.

DISCUSSION

The major finding of our study is that no adverse allergic reactions were recorded after ingestion of cheese containing lysozyme as compared with lysozyme-free cheese. Furthermore, lysozyme consumption with cheese did not determine an increase in specific immunoglobulins. Although the reduction of total IgA circulating levels was statistically significant (Table 3), the values were still in the normal range (2–3 mg/ml) and, therefore, no speculations on this reduction can be made.

In relation to the lack of effects of lysozyme on total or specific IgE, the presence of biogenic amines does not completely justify the results. In fact, the higher concentration of amines in lysozyme-free, 16-month aged cheese did not determine any difference in immunoglobulin levels when compared with lysozyme-containing cheese.

Lysozyme is a bacteriolytic enzyme and can affect the composition of cheese microflora. As a consequence the bacterial proteolysis can be modified, and this different proteolytic activity can affect the presence of allergenic

epitopes in cheese. Our preliminary results (Magnano San Lio, unpublished data) and a paper from Sforza et al. [34] support the hypothesis of a different peptidic pattern in cheese produced with or without lysozyme.

Lysozyme-sensitive subjects had higher levels of ovomucoid-specific IgE (Table 2), and this is probably the reason for the clear relationship observed between the level of total IgE and ovomucoid-specific IgE irrespective of the presence of lysozyme in the administered cheese (Table 6).

Given that neither cheese nor lysozyme should contain ovomucoid, this strong relationship could be explained by a cross-reaction between ovomucoid-specific IgE with peptides deriving from casein hydrolysis or with a contamination of lysozyme with ovomucoid. Everberg et al. [35] reported the presence of mixtures of egg protein in theoretically pure batches of albumin or ovomucoid but not of lysozyme. An immunologically relevant contamination with ovomucoid in commercial batches of lysozyme has been reported by Bernhisel-Broadbent et al. [36] and Jacobsen et al. [37]. In the experiment of Bernhisel-Broadbent et al. [36], performed on children, the major

Table 4. Extended

Location: Rome				
Basal Value T ₀	Ig Levels after 1 h (T ₁)			
	Without Lysozyme		With Lysozyme	
	A	B	C	D
3.75 ± 1.43	3.61 ± 1.31	3.53 ± 1.17	3.27 ± 1.12*	3.16 ± 0.98**
84.26 ± 66.03	83.78 ± 67.85	84.58 ± 73.92	80.62 ± 66.47	74.51 ± 70.22
0.216 ± 0.086	0.209 ± 0.081	0.221 ± 0.088	0.213 ± 0.097	0.219 ± 0.079
16.38 ± 10.18	16.40 ± 10.21	16.43 ± 10.18	15.67 ± 9.03	15.43 ± 10.88
1.81 ± 1.71	1.84 ± 1.75	1.82 ± 1.71	1.79 ± 1.67	1.80 ± 1.66

Table 5. Extended

Location: Campobasso			Location: Rome		
24 mo Aging			24 mo Aging	24 mo Aging	
With Lysozyme (D)	Without Lysozyme (B)	<i>p</i>	With Lysozyme (D)	Without Lysozyme (B)	<i>p</i>
62.11 ± 12.82	93.31 ± 20.34	0.0013	88.18 ± 16.22	96.45 ± 10.05	0.0443
79.81 ± 16.01	94.42 ± 14.04	0.0110	85.69 ± 17.22	97.61 ± 13.11	0.0005
100.57 ± 3.02	100.57 ± 2.78	0.8946	104.60 ± 12.44	102.18 ± 11.71	0.9669
99.59 ± 7.79	98.78 ± 5.44	0.1573	94.04 ± 23.06	100.57 ± 1.09	0.0680
98.47 ± 5.15	99.52 ± 7.40	0.6861	100.37 ± 3.85	100.87 ± 4.00	0.7329

factor responsible for the egg-white immunological response was the ovomucoid fraction. This could explain the high positive correlation between ovomucoid-specific and total IgE observed in our experiment.

According to Walsh et al. [38], egg-sensitized people can be divided into four groups: (1) with both lysozyme and ovalbumin allergies; (2) ovomucoid-only sensitization; (3) ovomucin sensitization, and (4) allergy to egg yolk. Walsh et al. [38] studied egg allergy in children; whereas, Langeland and Harbitz [39] and Ando et al. [40], in two studies involving adult subjects, reported a link between immunological response to ovomucoid and ovalbumin. However, our results do not support the results of Langeland and Harbitz [39] and Ando et al. [40].

CONCLUSION

In conclusion, Grana Padano cheese does not differ in terms of allergenicity from lysozyme-free TrentinGrana. Lysozyme does not seem to play a major role in the immunological response to cheese consumption.

Table 6. Correlation (*r* Coefficient) between Immunoglobulin Blood Levels in Egg-Sensitive Subjects, Having Different Lysozyme Sensitivity and Receiving Cheese of Two Different Aging Period (16 or 24 Months) and Containing or Not Containing Lysozyme

	16 mo (<i>r</i>)	24 mo (<i>r</i>)
Location: Termoli, Campobasso		
Without lysozyme		
IgE tot – IgE alb	0.670*	0.632†
IgE alb – IgE ovo	–0.646†	–0.653†
With lysozyme Si		
IgE tot – IgE alb	0.643†	0.657†
IgE alb – IgE ovo	–0.641†	–0.647†
Location: Rome		
Without lysozyme		
IgE tot – IgE alb	0.740**	0.650**
IgE tot – IgE ovo	0.954**	0.941**
IgE alb – IgE ovo	0.753**	0.775**
With lysozyme		
IgE tot – IgE alb	0.672**	0.677**
IgE tot – IgE ovo	0.868**	0.866**
IgE alb – IgE ovo	0.757**	0.659**
IgE lyso – IgA	0.532*	0.556*

tot = total; alb = albumin; ovo = ovomucoid; lyso = lysozyme.

* *p* < 0.05; ** *p* < 0.01; † *p* < 0.07.

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