RESEARCH ARTICLE

Lysozyme in wine: A risk evaluation for consumers allergic to hen's egg

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Lysozyme used in wine production could present a risk for consumers allergic to hen's egg. Thus, precautionary labeling of lysozyme on wines has been adopted within the European Community by updating Annex IIIa by Directive 2007/68/EC on November 27, 2007. Since no scientific data is known about the actual amounts and risks of lysozyme in wines, various *in vitro* efforts and skin prick tests were applied in this study to evaluate the presence of lysozyme in wines and the reactivity of those residues in allergic individuals and to fulfill the claim of updating Annex IIIa announced in Directive 2003/89/EC. Depending on the wine's color (red or white wine) and fining with bentonite, which is known as an important step to remove unstable proteins mainly from white wines, diverse results were obtained concerning the amounts of lysozyme in finished wines and their *in vitro* and *in vivo* reactivity in humans allergic to hen's egg.

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1 Introduction

Lysozyme is an enzyme derived from hen's egg white that is used in wine and cheese production for a better control of the fermentation process and against spoilage during production. Color loss is a dreaded aberration particularly in red wine production and can be prevented effectively with lysozyme. Thus, it is used to increase the effect and to lower the dosage of sulfites. Sulfites are commonly known to trigger adverse reactions in sensitive humans and are a major factor in wine intolerance [1]. Additionally, the unspecific antimicrobial activity of sulfites influences the yeast activity and flavor. Thus, lysozyme is an appreciated additive [2, 3] that can be used in dosages up to 50 g/hL within the European Community (EC).

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Abbreviations: EC, European Community; **MW,** molecular weight; **PMF,** peptide mass fingerprint; **SPT,** skin prick test; **TMB,** 3,3′,5,5′-tetramethylbenzidine

Besides the positive characteristics, lysozyme is a well-known major allergen from hen's egg named Gal d 4. It contains both conformational and sequential epitopes [4–7]. Allergic reactions exclusively to lysozyme were reported by Pérez-Calderón *et al.* [8], Malmheden [9], Frémont *et al.* [10], and Camp *et al.* [11] at lower milligram dosages in a double-blind placebo-controlled food challenge [10]. Because of its allergenicity, lysozyme must be labeled on wines if present in the final product, even if used as a processing aid. Labeling within the EC is regulated by Annex IIIa in Directive 2007/68/EC amending Directive 2000/13/EC and by other regulations in nations such as Australia, New Zealand, Japan, or the United States. However, only little information is available concerning the presence of lysozyme and the risk of amounts present in wines. Weber *et al.* found that the lysozyme was the only substance among various processing aids that could be detected *in vitro* in wines at common dosages [12]. With regard to the allergic potential of this finding and due to the lack of reliable lowest observed adverse effect levels for lysozyme, *in vivo* studies, and the influence of important technological processing steps not considered in that study, further investigations are necessary.

Some processing steps and interactions with matrix substances, particularly polyphenols, could lead to a signif-

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icant decrease of lysozyme in wine. Important processing steps could be the treatment of wines with bentonite or fining agents like casein or isinglass. Bentonite is a kaolin-like mineral that is commonly and preferentially used in wine processing to avoid protein clouding by protein adsorption [13]. Consequently, adsorbed proteins from grapes, yeast, or lysozyme are removed in the following filtration or decanting step [13–15]. A previous investigation of different German white wines treated with lysozyme and bentonite provided lysozyme residues between 0.01–0.06 ppm for initial dosages of 25 and 50 g/hL, respectively [12].

Fining agents, such as caseinate or isinglass, are used to reduce the amount of tannins and polyphenols in wines. Additionally, they are able to adsorb proteins due to electrostatic interactions. Thus, the usage of fining agents must be considered as a potential factor for the removal of lysozyme in wines. The fining agents themselves are removed during wine processing to a nondetectable amount from almost every wine [12].

To evaluate the effect of bentonite and fining agent treatment applied in wine production, we determined the lysozyme content and its antigenic property by HPLC, SDS-PAGE, and immunoblotting with sera from adults allergic to hen's egg. HPLC was applied in wines without bentonite treatment because higher amounts of lysozyme were assumed, and ELISA is suitable only for trace analysis ≤ 1 ppm [12]. These results were corroborated by MALDI-TOF MS/MS and skin prick tests (SPT). SPT are known for a high accuracy in forecasting the absence of IgE reactions *in vivo* and, consequently, the possibility of allergic reactions [16].

2 Materials and methods

2.1 Patients

Sera were collected from six adult patients (five women and one man with an average age of 49 years) allergic to hen's egg (Table 1). Patients suffered from atopic eczema ($n = 6$), allergic asthma ($n = 5$), and rhinoconjunctivits allergica ($n = 4$) with multiple sensitizations to foods. They had developed anaphylaxis (grade I–III according to Ring and Messmer [17]) with symptoms of urticaria, dyspnea, tachycardia, hypotension, and/or diarrhea together with an oral allergy syndrome, erythema, and pruritus. The allergy was confirmed by a SPT to a commercial egg white extract (Allergopharma, Reinbek, Germany) as well as by the demonstration of the specific IgE antibodies to egg white proteins in the radio allergosorbent test. One control serum was collected from an adult nonatopic and nonallergic to hen's egg.

2.2 Wine samples

Five different and chemically well-characterized German wines (year 2004) were prepared in cooperation with the Dienstleistungszentrum Ländlicher Raum (DLR) Mosel, Trier, Germany, according to a typical processing protocol: Riesling Mosel, Riesling Rheingau, Pinot blanc Pfalz, Pinot gris Baden (white wines), and Dornfelder Rheinhessen (red wine). Briefly, untreated wines were provided from different winemakers and stored in carboys. After cross-flow filtration, the wines were treated with dosages of the stabilizer lysozyme of 25 and 5 g/hL for a period ranging between 13 and 18 days. Both dosages were within the regulated thresholds as described in Section 1. Afterwards, the wines were filtered, treated with 200–700 g/hL bentonite and cross-flow filtrated. The bentonite dosage was determined with two industrial standard methods: Bentotest® and the caloric method at 65°C. The Bentotest® consists of a specific reagent mainly composed of phosphomolybdic acid and hydrochloric acid, which is added to the wine sample. Unstable wine proteins are detected due to the formation of haze caused by the cross linkage of molybdenum ions and proteins. Afterward, small samples of the unstable wine are treated with increasing bentonite dosages until no haze is detected in the Bentotest®. The caloric method depends on the formation of haze due to denaturation of unstable proteins by heating at 65°C and, afterward, cooling in the refrigerator. The bentonite dosage is determined as in the Bentotest® method. Results for the determined bentonite dosages are shown in Table 2. Finally, the wines were

Table 1. Clinical characteristics of patients

Patient ID	Gender	Age	Egg-specific RAST class	SPT wheal diameter to egg (mm)	Total IgE level (kU/L)	History of atopy
0	Female	49	0	<3	nd	None
1	Female	58	4	17	10,835	AE, AB, RCA
2	Female	52	5	19	3,401	AE, AB, RCA
3	Female	33	2	19	1,321	AE, AB, RCA
4	Female	58	5	13	4,864	AE, AB, RCA
5	Female	71	3	10	588	AE
6	Male	25	1	nd	nd	AE, AB

RAST classes correspond to hen's egg-specific IgE concentrations of 0 < 0.35 kU/L; 1, 0.35–0.7 kU/L; 2, 0.7–3.5 kU/L; 3, 3.5–17.5 kU/L; 4, 17.5–50 kU/L and 5, 50–100 kU/L. AE, atopic eczema; AB, allergic asthma bronchiale; RCA, allergic rhinoconjunctivitis allergica; nd, not determined.

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bottled through a membrane filter and sealed with a screw cap. For each wine, lysozyme-free control wines and wines without bentonite treatment were provided.

Lysozyme for wine treatment was provided by SIHA Begerow (Langenlonsheim, Germany). The white granulate was derived from hen's egg white and is recommended for the treatment of white and red wines in dosages between 15 and 30 g/hL by the manufacturer.

2.3 Caseinate and isinglass fining

Model wines treated with 25 g/hL lysozyme were treated either with potassium caseinate or with isinglass (wine fining agents from Erbsloeh, Geisenheim, Germany) according to the manufacturer's instructions. Therefore, 100 mg potassium caseinate was dissolved in 1 mL bidist. water while shaking. Two microliters of this solution was added to 2 mL of wine, corresponding to the medium recommended dosage of 10 g/hL. For isinglass, 50 μL isinglass solution was suspended in 500 μL of the respective wine and mixed thoroughly. Afterward, 10 μL of this solution was added to 2 mL wine, corresponding to the medium recommended dosage of 50 mL/hL. Wines treated with caseinate or isinglass were incubated and shaken for 2 days at 10°C. Finally, the wines were filtered through a membrane filter and the clear filtrate was stored at −80°C until further investigation by HPLC. No bentonite treatment was performed prior or afterward.

2.4 Wine sample preparation

Wines were centrifuged at 8500 × *g* for 10 min to remove residual clouding. The supernatant was stored at −80°C until further investigation.

A 100:1 concentration of wines was obtained by freeze-drying. To do so, 100 mL wine was freeze-dried and the residuum was dissolved in 12 mL bidist. water. The solution was injected to a Pierce 3500 MWCO dialysis chamber (Peribo Science, Bonn, Germany) and dialyzed at room temperature two to three times against 800 mL bidist. water for 48 h in the case of white wines and four days in the case of red wines. Afterward, the dialysate was freeze-dried again, dissolved in 1 mL of bidist. water and centrifuged at 8500 × *g* for 10 min. The supernatant was stored at −80°C.

2.5 Reagents, buffers, and instrumentation

All chemicals used were of analytical grade.

Analytical grade lysoyzme was purchased from Sigma (Sigma-Aldrich, Munich, Germany).

For immunostaining, the following solutions were prepared: The Tris solution contained 50 mM Tris, 150 mM NaCl, and 0.5% polyethylene-sorbitan monolaurate in bidist. water [18]. The 3,3′,5,5′-tetramethylbenzidine (TMB) solution consisted of 12 mg TMB and 40 mg dioctyl sodium sulfosuccinate dissolved in 10 mL ethanol. Citric buffer (pH 5.0) was prepared of 9.4 g citric acid and 18.2 g $Na_2HPO_4 \cdot 2H_2O$ dissolved in 1 L bidist. water. The used substrate solution was carefully mixed from 5 mL TMB solution, 15 mL citric buffer, and 10 μL H_2O_2 (30%). Rebuffer solution was prepared with 121.2 mg Tris, filled up to 80 mL with bidist. water, adjusted to pH 6.0 with 4 M hydrochloric acid, and finally filled up to 100 mL with bidist. water. Ponceau red solution was prepared of 2% Ponceau red and 3% trichloroacetic acid in bidist. water.

Polyclonal rabbit anti-human IgE immunoglobulins (order no. A0094), polyclonal goat anti-rabbit biotine-conjugated immunoglobulins (order no. E0432), and avidine/horseradish peroxidase conjugate (order no. P0347) were purchased from Dako (Hamburg, Germany).

2.6 SDS-PAGE

SDS-PAGE was performed on Invitrogen equipment (Invitrogen, Karlsruhe, Germany) using Invitrogen MES running buffer as electrolyte. The proteins were separated with 1-mm-thick, 8 × 8 cm, precast NuPAGE Novex gels with 12% acrylamide and bis–Tris buffer system (Invitrogen). Serva Mark12 (Invitrogen) was used as molecular weight (MW) marker. Electrophoresis was performed at 200 V for 45–50 min. Gels were either stained with CBB or were used for semidry Western blot and immunostaining.

2.7 Semidry Western blot and immunostaining

Proteins were transferred onto PROTRAN nitrocellulose transfer membranes with 0.2 μm pore size (Whatman, Dassel, Germany) by semidry Western blot for 80 min at 60 mA (one gel). For immunostaining, the air-dried

Table 2. Determined bentonite dosage for the assayed wines

Bentonite dosage for wines treated with	Riesling Mosel (g/hL)	Riesling Rheingau (g/hL)	Pinot blanc (g/hL)	Pinot gris (g/hL)	Dornfelder Rheinhessen (g/hL)
0 g/hL lysozyme	200	0	250	0	80
25 g/hL lysozyme	250	200	550	330	80
50 g/hL lysozyme	400	450	700	400	100

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membranes were blocked with Tris solution and incubated over night with patient sera (1:6 diluted in Tris solution). The next morning, the membranes were consecutively incubated with rabbit anti-human IgE immunoglobulins, diluted 1:1000 in Tris solution, goat anti-rabbit biotine-conjugated immunoglobulins, diluted 1:2000 in Tris solution, and avidin/horseradish peroxidase solution, and diluted 1:4000 in Tris solution. Afterwards, the membranes were incubated in rebuffer solution. Every step was followed by thorough washing with Tris solution. Finally, the membranes were incubated with substrate solution until the desired color intensity was obtained. The reaction was stopped by washing with bidist. water. The MW marker and lysozyme sample were stained by the addition of Ponceau red solution for 1.5 h followed by thorough washing with bidist. water.

2.8 MALDI-TOF MS-MS

Protein identification by MALDI-TOF MS-MS was performed according to the protocol published elsewhere [19]. Database searches were performed in the complete Swiss-Prot or National Center for Biotechnology Information nr (NCBInr) primary sequence databases. Only proteins represented by at least one peptide sequence above the significant threshold in MS/MS in combination with the presence of at least four peptide masses assigned in the peptide mass fingerprint (PMF) were considered as identified. The significant threshold was defined with $P = 0.05$.

2.9 HPLC

HPLC analysis was performed on Merck Hitachi equipment (Tokyo, Japan). Lysozyme was separated using a gradient program with solvents A and B. The initial ratio of A/B was 100/0 (%/%, v/v) for 8 min, followed by a 2 min ramp to A/B: 85/15. A/B: 85/15 was held for 5 min. Afterward, a ramp to A/B: 0/100 was performed for another 5 min and was held constant for 10 min. Finally, the initial ratio of A/B: 100/0 was reconstituted by a 5 min ramp and the column was conditioned for 10 min, giving a total run time of 45 min. Solution A consisted of 66.9% bidist. water, 33% acetonitrile, and 0.1% trifluoroacetic acid. Solution B was prepared from 99.7% acetonitrile and 0.3% formic acid. Lysozyme was detected with a retention time of 14.2 min.

Separation was performed with a flow rate of 1 mL/min on a Jupiter C-18 250 × 4.6 mm reversed phase column with a pore size of 300 Å and a particle size of 5 µm (Phenomenex, Aschaffenburg, Germany). Fluorescence was detected at 280 nm excitation and 340 nm emission [20].

This method was capable of detecting about 0.5 ppm lysozyme (limit of detection) with a linear range between 4 and 30 ppm. Variation coefficients were $< 10\%$ in this range for pure lysozyme. A 1:20 dilution of white wines and 1:2 dilution of Dornfelder red wine were necessary to fit within this range. Measurements were performed in triplicate.

2.10 Skin prick tests

Three patients (patient 1, 3, and 4 according to Table 1) were recruited for SPT. Tests were performed in duplicate on both volar forearms on two separate days with an assortment of lysozyme-treated and -untreated control wines. Bentonite treatment was considered for all five wines, whereas Dornfelder red wine was the only wine investigated with and without bentonite treatment in the SPT. Respective wines were tested undiluted and concerning bentonite-treated wines with a concentration of 100:1. Duplicate results are given as an average wheal diameter.

SPT were performed using a 1 mm single peak lancet and reactions were read after 20 min. Wheals larger than 3 mm with a surrounding flare were taken as positive SPT results [21]. Histamine dihydrochloride 0.1% served as a positive and bidist. water as a negative control. Pure lysozyme was skin tested in concentrations of 1 and 100 ppm in bidist. water.

3 Results

3.1 Electrophoresis with colloidal Coomassie staining

Analysis of commercially available lysozyme for wine treatment revealed four protein bands: 30, 15, 10, and 8 kDa (Fig. 1, lane A). By mass spectrometry, all bands were shown to represent the complete amino acid sequence (79, 91, 88, and 88% sequence coverage) of lysozyme. Hence, these bands are due to dimerization (30 kDa) or unknown nonprotein modifications. The major band of 10 kDa represents the main component. Lysozyme, however, is described with a MW of 14.4 kDa. This difference is most likely due to different conditions between the samples and the ready-to-use MW marker because analytical grade lysozyme migrates to the same position (Fig. 1, lane B).

Analysis of bentonite-treated wines revealed bands in the MW range of lysozyme (~10 kDa) only for wines treated with 50 g/hL lysozyme and a concentration of 100:1 (Fig. 1). Such bands were not detected in wines treated with 25 or 0 g/hL lysozyme and in nonconcentrated wines (results not shown). Seven different bands were detected in wines treated with 50 g/hL lysozyme and concentrated to 100:1. MWs were calculated with ~117 (Pinot blanc and Pinot gris), ~83 (weak band in all white wines), ~65 (Riesling Mosel, Pinot blanc, and Pinot gris), ~43 (weak band in Riesling Mosel, Pinot blanc, and Pinot gris), ~22 (Riesling Mosel and Pinot gris), ~16 (Pinot blanc), and ~10 kDa (weak band in Riesling Mosel, Pinot blanc, and Pinot gris). For Dornfelder, no clear protein bands could be obtained. Dornfelder red wine

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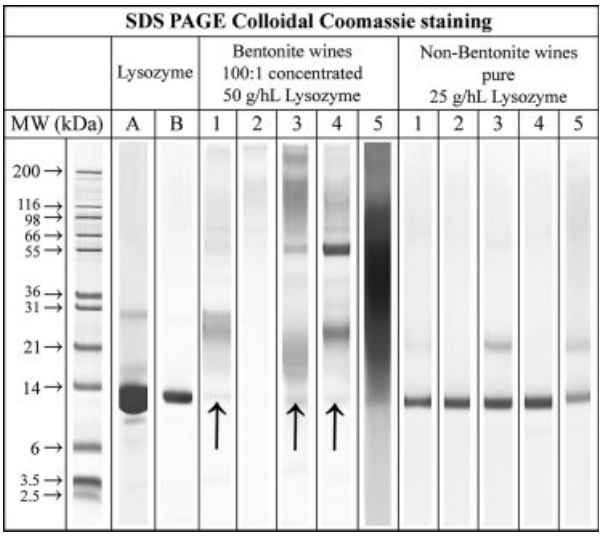

Figure 1. SDS-PAGE with colloidal Coomassie staining of lysozyme-treated wines. (A) lysozyme adjuvant for wine treatment; (B) analytical grade lysozyme. 1, Riesling Mosel; 2, Riesling Rheingau; 3, Pinot blanc; 4, Pinot gris and 5, Dornfelder Rheinhessen.

remained strongly colored despite dialysis for four days. Thus, matrix effects were considered. Lysozyme could be clearly identified in the MW range of 10 kDa in Pinot blanc, Pinot gris, and Dornfelder by mass spectrometry (sequence coverage 80, 85 and 80%; 10 of 29, 12 of 35, and 11 of 40 peptide matches found in PMF; two peptide sequences with identity signifying scores were obtained for each band by MS/MS).

In nonbentonite wines, bands with the MW of lysozyme were already revealed in wines treated with 25 g/hL lysozyme and without concentration. Figure 1 shows bands at ~20 (Riesling Mosel, Pinot blanc, and Dornfelder) and 10 kDa (all wines). The 10 kDa bands were identified as lysozyme by mass spectrometry (81–87% sequence coverage; 12 of 52 (Riesling Mosel), 13 of 51 (Riesling Rheingau), 13 of 44 (Pinot blanc), 14 of 54 (Pinot gris), and 11 of 40 (Dornfelder) peptide matches found in PMF; two peptide sequences with scores indicating identity were obtained by MS/MS) and by 2D electrophoresis (results not shown).

3.2 Electrophoresis with immunostaining

Initially, antibody binding to the lysozyme adjuvant for wine treatment was demonstrated for all used human sera. Every serum was capable of binding to the lysozyme adjuvant as shown in Fig. 2. Immunostaining revealed four different bands with MW of ~8–14 (all sera), ~28 and ~69 (five of six sera), and ~84 kDa (one of six sera).

Immunostaining of a 10 kDa band was observed for bentonite-treated Pinot blanc, Pinot gris, and Dornfelder at a dosage of 50 g/hL lysozyme and a concentration of 100:1 (Fig. 3). In Pinot blanc two sera, in Pinot gris four sera, and in Dornfelder three sera recognized the 10 kDa band. Interestingly, antibodies bound also and more intensively to proteins with MWs higher than lysozyme. Main protein bands in Pinot blanc and Pinot gris were calculated with MWs of ~57 and ~20 kDa. In Pinot gris, the 57 kDa protein was identified as vacuolar invertase 1 from *Vitis vinifera* by mass spectrometry (15 peptide matches in PMF; 2 peptide sequences obtained by MS/MS). Additionally, lysozyme was identified at ~57 kDa in Pinot blanc and Riesling Mosel. Database searches of the ~22 kDa bands indicated a mixture of different proteins or, better yet, fragments thereof. These are derived from two proteins: first, vacuolar invertase 1 (*Vitis vinifera*; no match in PMF; however, two peptide sequences identified in both Pinot blanc and Pinot gris) and second from VVTL1, a thaumatin-like protein from *Vitis vinifera* (also no protein match in PMF but two peptide sequences by MS/MS determined in Riesling Mosel, Pinot blanc, and Pinot gris). No lysozyme was detected in the 22 kDa bands. The MWs of intact vacuolar invertase 1 (~57 kDa) and VVTL1 (~20 kDa) agreed well with published data of 62 and 24 kDa, respectively [22].

Immunostained protein bands from Dornfelder red wine with MWs higher than lysozyme were calculated with MWs of ~66 and 40–20 kDa. No matches were found in database searches after mass spectrometry.

Immunostaining in the range of 10 kDa does not occur in bentonite wines with a lysozyme dosage of 25 g/hL and in wines without lysozyme and their 100:1 concentrates (results not shown).

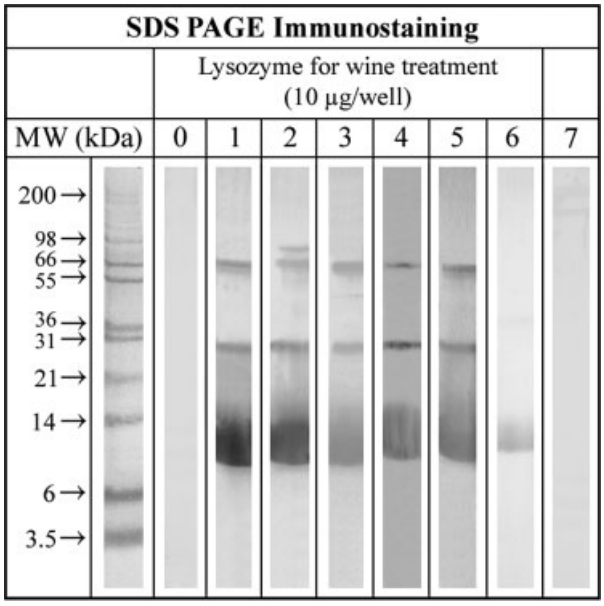

Figure 2. SDS-PAGE with immunostaining of lysozyme for wine treatment. 0, serum from nonallergic patient; 1–6, sera of patients allergic to hen's egg; 7, buffer control (without lysozyme). MW marker was stained with Ponceau red.

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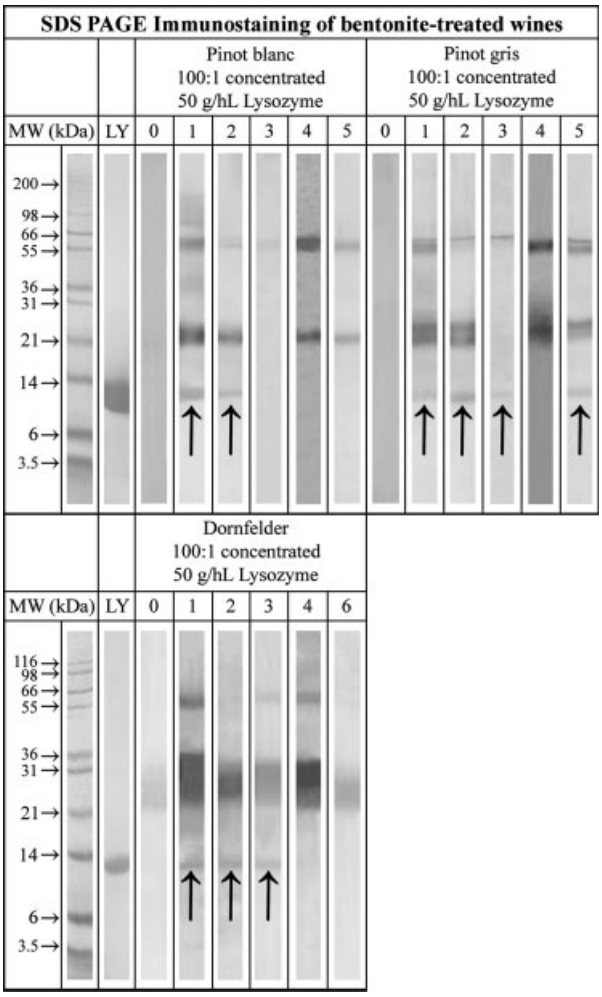

Figure 3. SDS-PAGE with immunostaining of bentonite-treated wines. LY, lysozyme adjuvant; 0, serum from nonallergic patient; 1–6, sera 1–6 of persons allergic to hen's egg. MW marker and lysozyme were stained with Ponceau red.

Immunostaining of wines without bentonite treatment was focused on Dornfelder red wine because bentonite treatment is essential for almost all white wines. The natural protein precipitating properties (due to the complex formation between proteins and wine polyphenols, such as condensed tannins and anthocyanins) and color reduction by bentonite discourage its usage in red wines. Dornfelder red wine revealed immunostaining of lysozyme at a MW of 10 kDa and, additionally, at ~22 and 55 kDa and, in one case, at ~28 kDa (Fig. 4). Except for the 10 kDa lysozyme band, all of these bands also occurred in Dornfelder without lysozyme treatment (results not shown).

3.3 Quantification of lysozyme in wines

Lysozyme was determined by ELISA in quantities between 0.001–0.006 g/hL in bentonite-treated white wines with dosages of 25 and 50 g/hL lysozyme [12].

Wines without bentonite treatment were found to contain significantly higher concentrations of lysozyme. HPLC analysis revealed amounts ranging between 12.3–18.3 $\pm$ 0.3–2.1 g/hL in white wines and 2.7 $\pm$ 0.3 g/hL in Dornfelder red wine for a lysozyme dosage of 25 g/hL (corresponding to 49.4–73.2% recovery for white wines and 10.9% recovery for Dornfelder red wine). Wines treated with 50 g/hL lysozyme were detected to contain 17.0–32.7 $\pm$ 0.2–0.7 g/hL in white wines and 3.8 $\pm$ 0.2 g/hL in Dornfelder red wine (corresponding to 34–65% recovery for white wines and 7.7% recovery in Dornfelder red wine).

3.4 Influence of caseinate and isinglass fining

HPLC analysis of lysozyme revealed mainly insignificant differences between wines fined with caseinate and isinglass and unfined wines. Unfined wines were found to contain lysozyme between 13.1–17.8 $\pm$ 0.1–0.2 g/hL (white wines) and 2.9 $\pm$ 0.1 g/hL (red wine), supporting our previous results. Fining with caseinate resulted in lysozyme amounts between 12.7–16.9 $\pm$ 0.1–0.7 g/hL (white wines) and 3.2 $\pm$ 0.3 g/hL (red wine). None of these findings indicated a significant difference to unfined wines by *t*-test.

Fining with isinglass resulted in lysozyme amounts between 12.6–16.4 $\pm$ 0.1–0.5 g/hL (white wines) and 3.1 $\pm$ 0.1 g/hL (red wine). Thereof, lysozyme contents of

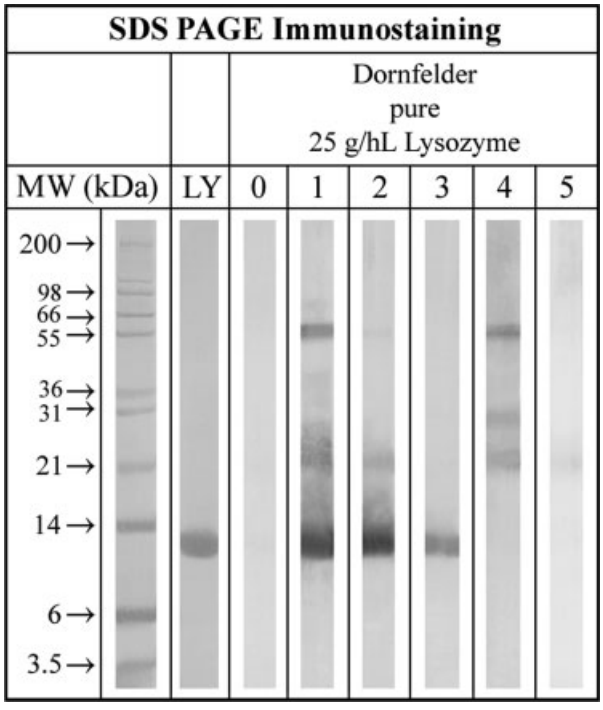

Figure 4. SDS-PAGE with immunostaining of Dornfelder red wine without bentonite treatment. LY, lysozyme adjuvant; 0, serum from nonallergic patient; 1–5, sera 1–5 of persons allergic to hen's egg. MW marker and lysozyme adjuvant were stained with Ponceau red.

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Riesling Rheingau (13 ± 0.1 g/hL), Pinot blanc (12.6 ± 0.1 g/hL), and Pinot gris (16.4 ± 0.1 g/hL) were significantly lower compared with unfined Riesling Rheingau (13.4 ± 0.1 g/hL), Pinot blanc (13.1 ± 0.1 g/hL), and Pinot gris (17.8 ± 0.2 g/hL).

3.5 Skin prick tests

Results for SPT are summarized in Table 3. Positive SPT against lysozyme were obtained for two patients to 100 ppm but not to 1 ppm lysozyme. There were no significant differences in the skin test responses between unconcentrated bentonite-treated wines treated with 50 g/hL lysozyme and lysozyme-free control wines. For 100:1 concentrated wines, a slight positive skin response was observed to lysozyme-treated Dornfelder red wine in patient 1. Similarly, patient 4 showed a slight increase in the skin test wheal diameter of 100:1 concentrated and lysozyme-treated Dornfelder. A more pronounced difference was observed for the 100:1 concentrated Pinot gris with an average wheal diameter of 5.5 mm for lysozyme-treated and 3.5 mm for lysozyme-free wine.

The SPT results for Dornfelder without bentonite treatment were significant. Two patients revealed positive skin reactions with average wheal diameters of 4 and 5.5 mm for wines treated with 25 g/hL lysozyme, as well as 4 and 6 mm for wines treated with 50 g/hL lysozyme, whereas SPT to lysozyme-free control wines were negative.

4 Discussion

The results of this study indicate that lysozyme may present a harmful adjuvant in wine processing for consumers allergic to hen's egg. However, a strong influence of bentonite treatment on the amount of lysozyme remaining in wine and on minimizing the risk for allergic reactions was found. Although the amount of lysozyme in bentonite-treated wines appears negligible, non-bentonite-treated wines contain lysozyme, eliciting immunological reactions in egg allergic patients *in vitro* and *in vivo*.

According to Table 2, lysozyme-treated white wines demand much higher amounts of bentonite than untreated white wines. Thus, lysozyme is almost removed in the bentonite fining step. Nevertheless, small lysozyme residues

Table 3. SPT results for various wines in three egg-allergic patients

Wine (g/hL) and lysozyme (ppm) dosage	Average wheal diameter (mm)		
	Patient 1	Patient 3	Patient 4
Lysozyme 1	2	2	0
Lysozyme 100	**7.5**	**4.5**	2.5
Bentonite wines			
RM 50	1.5	2.5	2
RM 0	0.5	2	1
RM 50 (100:1)	2.5	**3.5**	**5.5**
RM 0 (100:1)	1.5	**4**	**5**
RR 50	2	2	2
RR 0	2.5	**3.5**	0.5
RR 50 (100:1)	1.5	**4**	**3**
RR 0 (100:1)	2	**4**	**4**
PB 50	2	2.5	1
PB 0	**3**	2.5	1
PB 50 (100:1)	1.5	**3.5**	**3.5**
PB 0 (100:1)	1.5	**3.5**	**4.5**
PG 50	2.5	1	1.5
PG 0	1.5	1	2.5
PG 50 (100:1)	2.5	**5**	**5.5**
PG 0 (100:1)	1	**4**	**3.5**
DF 50	2	1	2.5
DF 0	1	1	1
DF 50 (100:1)	**3.5**	**3.5**	**4.5**
DF 0 (100:1)	2	**4**	3
Nonbentonite wine			
DF 50	**6**	**4**	2
DF 25	**5.5**	**4**	2
DF 0	0.5	2	2

Bold numbers indicate positive results (wheal diameter ≥3 mm). RM, Riesling Mosel; RR, Riesling Rheingau; PB, Pinot blanc; PG, Pinot gris; DF, Dornfelder; 100:1, 100:1 concentrated wines; 1, 3, and 4, Patients according to Table 1.

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between 0.001 and 0.006 g/hL were detected in wines treated with 25 and 50 g/hL lysozyme [12]. *In vitro*, antibodies from sera of humans allergic to hen's egg reacted with these residues if dosages of 50 g/hL had been used and if the wines were concentrated to 100:1 (according to Fig. 3). Most sera recognized lysozyme in Pinot gris. This is in good accordance with our previous report since Pinot gris was found to contain the highest amount of residual lysozyme, about 0.006 g/hL [12]. Even Dornfelder red wine showed antibody-binding properties of the remaining lysozyme. This was surprising since red wines are known for their natural protein-precipitating properties as described previously. Thus, lower amounts of lysozyme in comparison to white wines were expected. This was proven by HPLC analysis in nonbentonite wines resulting in lysozyme amounts 4.5- to 8.6-fold lower in comparison to white wines.

A concentration of 100:1 was necessary in all bentonite-treated wines to observe antibody binding against the maximum allowed lysozyme dosage of 50 g/hL *in vitro*. No antibody binding could be detected in wines with a medium dosage of 25 g/hL lysozyme. *In vivo*, no significant skin reactions to undiluted and bentonite-treated wines were observed. A concentration of 100:1 was necessary to provoke slight differences in skin response in patient 1 and 4 for a total of three wines, but immunological activity against lysozyme appeared not to be an explanation in the latter case. Patient 4 showed positive skin reactions to the same, but lysozyme-free control wines. Furthermore, even 100 ppm of pure lysozyme, which is about 17-fold higher than the lysozyme amount in these wines (detected by ELISA) [12], did not trigger a positive SPT in this patient. Considering the highly atopic status of the patients, other proteins contained in wines, such as bacterial or yeast products, or cross-reacting proteins (e.g., cross-reacting carbohydrate determinants) could have yielded the positive SPT to lysozyme-free control wines. In addition, unspecific test reactions may occur in the SPT with unstandardized matrices, such as wine, especially in patients with long-standing atopic eczema, as has been described to orange juice in 58% of tested patients [23]. Conclusively, lysozyme-treated wines that were fined with bentonite represent a negligible risk in our *in vitro* investigations and SPT for consumers allergic to hen's egg. This applies for all dosages allowed within the EC.

Investigation of wines without bentonite treatment revealed that about 34–73% of lysozyme remains in white wines and about 10% in the assayed red wine. Thus, a medium lysozyme dosage of 25 g/hL in nonbentonite wines led to lysozyme residues more than 2000-fold (white wines) and about 450-fold (red wine) higher compared with bentonite-treated wines with 50 g/hL dosage. Therefore, even pure red wine treated with 25 g/hL lysozyme showed strong antibody binding due to residual lysozyme *in vitro* (Fig. 4). *In vivo* results were also alarming, providing clear immunological reactions in both patients with sensitizations to 100 ppm lysozyme against the assayed red wine without bentonite treatment. Fining with common fining agents such as caseinate or isinglass was proven to have no meaningful impact on the removal of lysozyme from wines. The highest observed significant mean adsorption of lysozyme was accomplished by isinglass in Pinot gris with an 8.1% decrease of lysozyme. Thus, the adsorption of caseinate and isinglass could be neglected compared with the observed strong *in vitro* and *in vivo* reactions.

The finding of about 10% residual lysozyme in red wine represented a mean value compared with findings from other research groups in red wines with 20% [15], 14.4%, and only traces [20]. Consequently, omitting the bentonite treatment leads to significant amounts of lysozyme in red wines. These amounts were proven to be a harmful risk in our *in vitro* investigations and SPT for consumers allergic to hen's egg. White wines without bentonite treatment were determined to contain considerably higher residues of lysozyme (investigated by HPLC) and presented a higher risk in *in vitro* and *in vivo* experiments (results not shown). Meta-tartaric acid is an adsorbent for lysozyme and should be considered for lowering the lysozyme content if bentonite treatment is not desired [24]. The usage of meta-tartaric acid is, however, restricted to 10 g/hL within the EC.

According to Directive 2003/89/EC, the use of lysozyme must be labeled on wines if still present in the final product. The presence of lysozyme was proven in this and our previous study [12] for all wines, whether treated with bentonite or not. However, Article 1 Paragraph ''f'' of Directive 2003/89/EC claims the systematical reexamination and update of Annex IIIa on the basis of recent scientific knowledge. According to our results, it seems not reasonable to label all wines that contain traces of lysozyme. Only those wines that present a real risk to the sensitive consumer should be marked. A general labeling involves great disadvantages. It leads to an unnecessary uncertainty and deterrent and, finally, to an avoidable limitation of the freedom of choice and reduction of the quality of life for allergic consumers. Thus, it is demanded more and more to avoid a general precautionary labeling because it does not help the sensitive consumer. Lysozyme has gained increasing importance for the wine industry and the demand has risen nearly threefold within the last 5 years (unpublished data of a lysozyme producer). Thus, labeling of lysozyme according to Annex IIIa of Directive 2007/68/EC on wines should be reconsidered, particularly in terms of a labeling exception for lysozyme-treated, but bentonite-fined wines represented by the vast majority of lysozyme-treated white wines. Therefore, further research involving oral challenges is needed.

Another interesting finding in this study was the occurrence of immunostaining of wine proteins with sera from patients allergic to hen's egg (Figs. 3 and 4). Similar findings were also found *in vivo* with positive SPT to all tested lysozyme free, 100:1 concentrated wines in patient 3 and 4. Regarding the high levels of total serum IgE in these patients (588–10 835 kU/L), multiple sensitizations to

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various environmental proteins and, thus, also to wine proteins or associated cross-reacting carbohydrate determinants may be a possible explanation. Immunostaining was observed with patient's sera regardless of lysozyme treatment, but not in the nonallergic control serum. Supportingly, the intensity of immunostaining to wine proteins seems to correlate with the total IgE levels. Figures 3 and 4 revealed the weakest immunostaining of ~57 and ~20 kDa wine proteins with sera 3 and 5 (total IgE levels of 1321 and 588 kU/L), whereas sera 1, 2, and 4 (total IgE levels of 10835, 3401, and 4861, respectively) showed the strongest staining of the respective wine proteins. Otherwise, cross-reactivities between wine and egg white proteins maybe an explanation but actual cases of allergic reactions because of such a cross-reactivity have not been reported. Sequence comparison between identified wine proteins VVTL1 and vacuolar invertase 1 from *Vitis vinifera* with the important egg allergens ovalbumin, ovomucoid, lysozyme, conalbumin, and ovomucin revealed no significant sequence identities (NCBI protein blast database search). With respect to 3-D epitopes, no comment is possible because 3-D structures of wine proteins are currently unknown. Thus, cross-reactivities between wine and egg white proteins appear unlikely. So, the actual cause and the relevance of the observed immunologic reactions remain unclear.

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