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SCIENTIFIC OPINION

Scientific Opinion related to a notification from the Oenological Products and Practices International Association (OENOPPIA) on lysozyme from hen’s egg to be used in the manufacture of wine as an anti-microbial stabilizer/additive pursuant to Article 6, paragraph 11 of Directive 2000/13/EC – for permanent exemption from labelling

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a scientific opinion related to a notification from the Oenological Products and Practices International Association (OENOPPIA) on lysozyme from hen’s egg used in the manufacture of wine as an anti-microbial stabilizer/additive pursuant to Article 6, paragraph 11 of Directive 2000/13/EC – for permanent exemption from labelling. Allergic sensitisation against lysozyme is common among egg allergic individuals. In winemaking, lysozyme is used for the control of lactic acid bacteria, and it is considered essential to obtain consistent and high quality. Lysozyme can be used at different stages of wine production and at different doses, and no steps are taken specifically to remove lysozyme from wine. In the studies provided by the applicant, lysozyme was detected in some of the lysozyme-treated wines under the proposed conditions of use. The applicant estimated lysozyme content in white wines with and without bentonite treatment, and in red wines without bentonite treatment. Residual amounts of lysozyme considered sufficient to trigger allergic reactions in susceptible individuals have been demonstrated in wines treated with lysozyme, and a number of clinical reports (including one double-blind placebo-controlled food challenge with lysozyme) described clinical allergic reactions to lysozyme. The Panel concludes that wines treated with lysozyme may trigger adverse allergic reactions in susceptible individuals under the conditions of use proposed by the applicant. © European Food Safety Authority, 2011

KEY WORDS

Wine, anti-microbial stabilizer, lysozyme, food allergy.

1 On request from the European Commission, Question No EFSA-Q-2011-00833, adopted on 15 September 2011.
2 Panel members: Carlo Agostoni, Jean-Louis Bresson, Susan Fairweather-Tait, Albert Flynn, Ines Golly, Hannu Korhonen, Pagona Lagiou, Martinus Løvik, Rosangela Marchelli, Ambroise Martin, Bevan Moseley, Monika Neuhäuser-Berthold, Hildegard Przyrembel, Seppo Salminen, Yolanda Sanz, Sean (J.J.) Strain, Stephan Strobel, Inge Tetens, Daniel Tomé, Hendrik van Loveren and Hans Verhagen. Correspondence: nda@efsa.europa.eu
3 Acknowledgement: The Panel wishes to thank the members of the Working Group on Allergy: Pagona Lagiou, Martinus Løvik, Rosangela Marchelli, Martin Stern, Stephan Strobel, Hendrik van Loveren, Jean Michel Wal for the preparatory work on this scientific opinion.

SUMMARY

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a scientific opinion related to a notification from Oenological Products and Practices International Association (OENOPPIA) on lysozyme from hen’s egg used in the manufacture of wine as an anti-microbial stabilizer/additive pursuant to Article 6, paragraph 11 of Directive 2000/13/EC – for permanent exemption from labelling.

Prevalence of allergy to egg proteins has been reported to be around 0.3 % in adults. Taking into account that egg allergic individuals can react to lysozyme and lysozyme-containing foods, it is appropriate for the Panel to assess the likelihood of adverse reactions in allergic individuals consuming products where lysozyme has been added during the manufacturing process.

In winemaking, lysozyme is used for the control of lactic acid bacteria. Lysozyme is allowed for use in food manufacturing (cheese and wine) in EU countries, and must follow purity specifications set forth in European legislation. The purity of only one commercial product was described in the application.

Lysozyme can be used at different stages of wine production and at different doses, and no steps are taken specifically to remove lysozyme from wine. In the studies provided by the applicant, lysozyme was detected in some of the lysozyme-treated wines under the proposed conditions of use.

The applicant stated that lysozyme is the weakest allergen among the four major egg white proteins and indicated a frequency of sensitisation to lysozyme among egg allergic subjects of 15 %, as compared to 53 % for ovotransferrin and 32 % for ovomucoid and ovalbumin. The Panel notes that IgE anti-lysozyme antibodies as markers of sensitisation have been found more often in other studies e.g. in 35 %, 53 %, and 100 % of egg allergic consumers.

The applicant cited two human studies in egg-allergic individuals undergoing skin prick testing with lysozyme-treated wines. The Panel notes that the results from these studies are consistent with the analytical findings of significant residual amounts of lysozyme in treated wines but provide no information about the clinical reactivity of egg-allergic individuals to wines treated with lysozyme when consumed orally.

The applicant acknowledged that lysozyme residues are present in lysozyme-treated wines and that lysozyme is a sensitizer. However, the applicant proposed that oral consumption of lysozyme may not elicit clinical allergic reactions in egg-allergic individuals. The Panel notes that reports (including one double-blind placebo-controlled food challenge, DBPCFC) of allergic reactions to lysozyme and lysozyme-containing foods among egg-allergic individuals are available in the literature, and that results from a clinical study on lysozyme-containing cheese do not allow conclusions about the safety of lysozyme consumption in clinically egg allergic individuals.

The Panel took into account that allergic sensitisation to lysozyme is common among egg allergic individuals, that residual amounts of lysozyme considered sufficient to trigger allergic reactions in susceptible individuals have been demonstrated in wines treated with lysozyme, and that a number of clinical reports (including one DBPCFC with lysozyme) described clinical allergic reactions to lysozyme.

The Panel concludes that wines treated with lysozyme may trigger adverse allergic reactions in susceptible individuals under the conditions of use proposed by the applicant.
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BACKGROUND

Article 6, paragraph 11 of Directive 2000/13/EC\(^4\) establishes the cases and conditions for amending Annex IIIa to that Directive, which includes a list of food ingredients or substances known as likely to trigger allergic reactions in sensitive individuals. It also sets up a procedure for exempting from labelling, under certain conditions, derivatives of these ingredients.

Pursuant to the procedure referred to above, a list of ingredients or substances derived from ingredients listed in Annex IIIa has been adopted by the Commission and is included in the Annex to Commission Directive 2007/68/EC\(^5\) of 27 November 2007, amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. Applicants who are seeking the exclusion of a given product from Annex IIIa have to submit a request, completed with the results of relevant scientific studies.

Therefore, in the context of the permanent labelling exemption procedure, the European Food Safety Authority is asked to provide scientific opinions on submissions in accordance with the present terms of reference.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29(1)(a) of Regulation (EC) N° 178/2002, the European Commission requests the European Food Safety Authority to evaluate the scientific data submitted by the Oenological Products and Practices International Association (OENOPPIA) in the framework of the procedure laid down in Article 6, paragraph 11 of Directive 2000/13/EC. On the basis of that evaluation, EFSA is requested to issue an opinion on the information provided, and particularly to consider the likelihood of adverse reactions triggered in susceptible individuals by the consumption of the following ingredients/substances used under the conditions specified by the applicant: lysozyme from hen’s egg to be used in the manufacture of wine as an anti-microbial stabilizer/additive.

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ASSESSMENT
Prevalence of allergy to egg proteins has been reported to be around 0.3% in adults (EFSA, 2004; Sampson, 2004; Sicherer and Sampson, 2010; Vierk et al., 2007). Taking into account that egg allergic individuals can react to lysozyme and lysozyme-containing foods (EFSA, 2005), it is appropriate for the Panel to assess the likelihood of adverse reactions in allergic individuals consuming products where lysozyme has been added during the manufacturing process.

Dossiers submitted by Deutscher Weinbauverband (DWV) and the Office National Interprofessionnel des Fruits, des Légumes, des Vins et de l’Horticulture (VINIFLHOR), and by the Winemakers’ Federation of Australia (WFA) and the Australian Wine Research Institute (AWRI), to the European Commission pursuant to Article 6, Paragraph 11 of Directive 2000/13/EC as amended by Directive 2003/89/EC for permanent exemption from labelling were the basis for earlier assessments of egg products and albumin (egg white), with or without lysozyme, used as fining agents in wine by the Panel on Dietetic Products, Nutrition and Allergies (NDA) (EFSA, 2007a, 2007b). A dossier submitted by the Association of Manufacturers of Natural Animal-derived Food Enzymes (AMAFE) was the basis for an early assessment of egg lysozyme used as additive in food (EFSA, 2005).

The present opinion is based on a dossier from the Oenological Products and Practices International Association (OENOPPIA), with an application for permanent exemption. This application refers to the use of lysozyme from hen’s egg in the manufacture of wine as an anti-microbial stabilizer/additive.

1. Characterisation of the fining agent
The common name is ‘egg white lysozyme’, the Commission on Enzymes of the International Union of Biochemistry’s systematic name is “peptidoglycan N-acetylmuramoylhydrolase”, its systematic number is EC No. 3.2.1.17, and its Chemical Abstract Service (CAS) number is 9001-63-2. In the official allergen nomenclature, egg lysozyme is Gal d 4. The E number of lysozyme is E1105.

Lysozyme is a non-glycosylated antimicrobial protein consisting of 129 amino acids, and has a molecular weight of about 14.4 kD. Lysozyme was the first protein to be sequenced and to have its three-dimensional structure completely analysed. Lysozyme from different sources shows only small variations in amino acid sequence and three-dimensional structure. No IgE binding epitope has yet been defined on lysozyme.

In winemaking, lysozyme is used for the control of lactic acid bacteria, and according to the applicant it is considered essential in certain situations to obtain consistent and high quality. Lysozyme is allowed for use in food manufacturing (cheese and wine) in EU countries, and must follow purity specifications set forth in European legislation. Lysozyme is extracted from egg white, where it constitutes approximately 0.3% of the mass and 3.5% of the proteins.

Lysozyme is produced under good manufacturing practice (GMP) conditions by the applicant. According to the applicant, the protein purity of their product is controlled by SDA-PAGE and HPLC. References are given to a report comparing the purity of the (industrial) lysozyme with a standard of “highly purified” lysozyme (Sigma-Aldrich ref. L6876). In SDS-PAGE, lysozyme appears “almost pure”, with only faint traces of avidin and of a lysozyme dimer, while gallin, conalbumin, ovalbumin, or ovomucoid were not detected. According to the applicant, avidin is not considered to be an egg allergen (Cooper et al., 2009). HPLC analysis shows that at a concentration of 10 g/L, contaminating egg proteins (conalbumin, ovomucoids and ovalbumin, the major egg allergens according to the

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applicant) have been reduced to undetectable levels at a limit of detection (LOD) of 10 mg/L for avidin and conalbumin, and 20 mg/L for ovalbumin.

The Panel notes that the purity of only one commercial product was described in the application.

2. **Conditions of use**

Lysozyme is used as an alternative to, or in combination with, sulphites to control growth of lactic acid bacteria without inhibiting the yeasts responsible for alcoholic fermentation. Specifically, the applicant listed four major applications for lysozyme: a) preventive control of the onset of malo-lactic fermentation (early addition of 100–150 mg/L); b) total inhibition of malo-lactic fermentation (at a dose of 500 mg/L); c) protection of wine during sub-optimal alcoholic fermentation (at doses of 250-300 mg/L); d) stabilization of wine after malo-lactic fermentation (at doses of 250-300 mg/L).

The Panel notes that lysozyme can be used at different stages of wine production and at different doses, and that no steps are taken specifically to remove lysozyme from wine.

3. **Analysis of residual allergens in wine**

In published literature cited by the applicant (Weber et al., 2007; 2009), residual amounts of lysozyme were detected in four German wines experimentally treated with lysozyme and fined with bentonite at the level of detection of the competitive ELISA used (LOD=0.001 mg/L), corresponding to 0.01 mg/L considering dilution. One wine had significantly higher levels of lysozyme (estimated at 0.06 mg/L). Without bentonite fining, the levels of lysozyme detected in wines by HPLC analysis were significantly higher (i.e. up to 183 mg/L and 327 mg/L in white wines treated with 250 mg/L and 500 mg/L of lysozyme, respectively, and up to 27 mg/L and 38 mg/L in red wines treated with 250 mg/L and 500 mg/L of lysozyme, respectively). The authors concluded that allergic reactions to lysozyme-treated wine cannot be excluded and that lysozyme-treated wine could possibly trigger allergic reactions in susceptible individuals after moderate wine consumption (0.1–0.7 L). The Panel notes that bentonite treatment is not mandatory in the manufacture of wine.

In a new study commissioned by the applicant, lysozyme residues were determined in 29 commercial lysozyme-treated wines (three white, two rosé, and 24 red wines) from five countries (vintages 2004-2010), with or without bentonite and metatartaric acid fining (Restani, 2011, unpublished). When examined unconcentrated by immunoblotting with an anti-lysozyme antibody, two wines showed lysozyme residues of 8.6 mg/L and 2.6 mg/L, respectively, which were above the reported limit of detection/quantification of 0.49 mg/L. The positivity for lysozyme was confirmed by HPLC (LOD=0.18 mg/L), by a microbiological plating method, and by the use of an anti-egg white antibody in immunoblotting (LOD=0.25 mg/L). When concentrated five times, three more wines showed residues of lysozyme close to the detection limit, suggesting an (unconcentrated) level of 0.1 mg/L lysozyme in these wines.

The Panel notes that lysozyme was detected in some of the lysozyme-treated wines under the conditions of use proposed by the applicant.

4. **Estimated level of exposure**

On the basis of the data reported by Weber et al. (2007; 2009), the applicant estimated lysozyme content in white wines with (0.06 mg/L) and without (200-330 mg/L) bentonite treatment, and in red wines without bentonite treatment (30-40 mg/L). The applicant provided exposure estimates to lysozyme from cheese and wine for 19 European countries, assuming a mean lysozyme content of 250 mg/kg in cheese and of 40 mg/L in wine. The Panel notes that intake on a single occasion may be more relevant regarding food allergic reactions than average daily or yearly intake, that no estimations
of lysozyme intakes on a single occasion have been provided, and that no generally applicable threshold levels of intake have been defined for food allergens (NDA, 2004).

5. Evidence of non-allergenicity

5.1. History of non-allergenicity of the product

The applicant stated that lysozyme is the weakest allergen among the four major egg white proteins (Bianchi, 1982; Hoffman, 1983; JECFA, 1993) and indicated a frequency of sensitisation to lysozyme among egg allergic subjects of 15%, as compared to 53% for ovotransferrin and 32% for ovomucoid and ovalbumin (Poulsen et al., 2001). The Panel notes that IgE anti-lysozyme antibodies as markers of sensitisation have been found more often in other studies e.g. in 35%, 53%, and 100% of egg allergic consumers (Fremont et al., 1997; Holen and Elsayed, 1990; Suzuki et al., 2010).

The applicant cited two human studies in egg-allergic individuals undergoing skin prick testing with lysozyme-treated wines.

One publication (Kirschner et al., 2009) reported on five patients allergic to egg (four with a positive and one with a negative skin prick test to lysozyme), three of whom underwent skin prick testing with two lysozyme-treated wines. Two patients reacted to the lysozyme-treated wines. However, one of these two patients also reacted to the corresponding control (unfined) wines. Different from what is stated in the application, this publication does not report on a double-blind placebo-controlled food challenge (DBPCFC) using lysozyme-treated wines. The Panel considers that these results are consistent with the analytical findings of significant residual amounts of lysozyme in treated wines but provide no information about the clinical reactivity of egg-allergic individuals to wines treated with lysozyme when consumed orally.

In the second publication (Weber et al., 2009), two out of three egg-allergic patients showed significant skin prick reactions to lysozyme (250 and 500 mg/L) treated red wine (unconcentrated, no bentonite treatment) and slight reactions to bentonite-treated but (100x) concentrated white and red wines (the same wines unfined were used as control). No oral challenge was performed. The Panel notes that these results are consistent with the analytical findings of significant residual amounts of lysozyme in treated wines but provide no information about the clinical reactivity of egg-allergic individuals to wines treated with lysozyme when consumed orally.

The applicant acknowledges that lysozyme residues are present in lysozyme-treated wines and that lysozyme is a sensitizer. However, the applicant proposes that oral consumption of lysozyme may not elicit clinical reactions in egg-allergic individuals. Accordingly, a literature search was performed by the applicant to identify possible cases of allergic reactions to lysozyme or lysozyme-containing foods (not limited to wine) in egg-allergic individuals. The strategy used and the database(s) searched were not described in the application. The applicant’s search identified two publications which describe possible allergic reactions to lysozyme (Fremont et al., 1997; Malmheden Yman, 2004).

One publication reported on a DBPCFC study (Fremont et al., 1997) in which two out of six egg allergic children showed clinical signs upon oral challenge with lysozyme. One additional patient had a labial challenge with one drop of lysozyme solution (1 mg/mL) and reacted. The applicant argues that insufficiencies in reporting (e.g. regarding the purity of the lysozyme preparations and the doses used) may limit the conclusions which can be drawn from this study with respect to the allergenicity of lysozyme.

The second publication described five cases of serious allergic reactions to lysozyme-treated cheese that were reported to the Swedish register along with 16 other cases of clinical reactions to egg (Malmheden Yman, 2004). The applicant stated that insufficiencies in reporting (e.g. presence of
other allergens in cheese, lack of data on lysozyme concentrations in the cheese samples) may limit the conclusions which can be drawn from this publication with respect to the allergenicity of lysozyme. The Panel is aware of a number of other case reports on allergic reactions supposedly triggered by lysozyme in cheese and in pharmaceutical products (e.g. Artesani et al., 2008; Ledesma Benitez et al., 2007; Perez-Calderon et al., 2007; Pichler and Campi, 1992).

The applicant also cites a single blind, placebo-controlled study (Iaconelli et al., 2008) in which 20 “egg allergic” subjects (17 of which had a radioallergosorbent test (RAST) positive for lysozyme and/or ovomucoid and ovalbumin) were challenged with up to 60 g of Grana Padano cheese containing 155 mg/kg lysozyme. No symptoms were triggered by the cheese in any of the challenged subjects. The Panel notes that apart from the statement that the “egg allergic” subjects had never experienced any gastrointestinal or respiratory symptoms on exposure to hen’s egg, no clinical characterisation or verification of clinical egg allergy was provided besides seropositivity. The Panel considers that this study does not allow conclusions to be drawn regarding the safety of lysozyme consumption in clinically egg allergic individuals.

The Panel notes that reports (including one DBPCFC) of allergic reactions to lysozyme and lysozyme-containing foods among egg-allergic individuals are available in the literature, and that results from one study using lysozyme-containing cheese are inconclusive with respect to the likelihood of clinical allergic reactions to oral consumption of lysozyme in egg-allergic subjects due to the inadequate clinical characterisation of the study population.

The Panel further notes that reactions to wine, including allergic reactions, are well documented (Armentia, 2008; Vally and Thompson, 2003). Since consumers and health professionals may be unaware that egg-derived products including lysozyme are used in the winemaking process, allergic reactions to lysozyme have generally not been considered following reactions to wine and therefore underreporting of reactions caused by lysozyme after ingestion of wines may have occurred.

5.2. Animal studies

No data on animal studies were provided in the application.

5.3. Clinical studies

No clinical studies were provided by the applicant besides those described in section 5.1.

CONCLUSIONS

The Panel took into account that allergic sensitisation to lysozyme is common among egg allergic individuals, that residual amounts of lysozyme considered sufficient to trigger allergic reactions in susceptible individuals have been demonstrated in wines treated with lysozyme, and that a number of clinical reports (including one DBPCFC with lysozyme) described clinical allergic reactions to lysozyme in egg-allergic individuals.

The Panel concludes that wines treated with lysozyme may trigger adverse allergic reactions in susceptible individuals under the conditions of use proposed by the applicant.

DOCUMENTATION PROVIDED TO EFSA

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EFSA (European Food Safety Authority), 2007a. Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from DWV and VINIFLHOR on egg products used as fining agents in wine pursuant to Article 6, paragraph 11 of Directive 2000/13/EC - for permanent exemption from labelling. The EFSA Journal, 567, 1-7.

EFSA (European Food Safety Authority), 2007b. Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from WFA and the AWRI on albumin (egg white) used in the manufacture of wine pursuant to Article 6, paragraph 11 of Directive 2000/13/EC - for permanent exemption from labelling. The EFSA Journal, 566, 1-7.


**GLOSSARY / ABBREVIATIONS**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AMAFE</td>
<td>Association of Manufacturers of Natural Animal-derived Food Enzymes</td>
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<td>AWRI</td>
<td>Australian Wine Research Institute</td>
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<td>CAS</td>
<td>Chemical Abstract Service</td>
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<td>DBPCFC</td>
<td>Double-blind placebo-controlled food challenge</td>
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<td>DWV</td>
<td>Deutscher Weinbauverband</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<tr>
<td>OENOPPIA</td>
<td>Oenological Products and Practices International Association</td>
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<tr>
<td>RAST</td>
<td>Radioallergosorbent test</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<td>WFA</td>
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