



Human IgE binding and *in vitro* digestion of S-OVA

Rodrigo Jiménez-Saiz, Carlos Pineda-Vadillo, Rosina López-Fandiño, Elena Molina*

Institute of Food Science Research (CIAL) CSIC-UAM C/Nicolás Cabrera, 9 28049 Madrid, Spain

ARTICLE INFO

Article history:

Received 12 March 2012

Received in revised form 9 May 2012

Accepted 8 June 2012

Available online 29 June 2012

Keywords:

S-ovalbumin

Ovalbumin

In vitro gastro intestinal digestion

IgE-binding

ABSTRACT

S-OVA, a more thermostable form of ovalbumin (OVA), was formed from native OVA or egg white *in vitro*, by heating at high pH, and by storage at low temperatures. S-OVA showed a much lower reactivity against IgE than OVA, although this difference in IgE binding was minimized after simulated gastro intestinal digestion, despite S-OVA was more resistant to proteolysis, particularly to pepsin, than its native form. It is, therefore, likely that the transformation of OVA to S-OVA does not affect its ability to sensitise or trigger allergic reactions at the duodenal level. These results are discussed in the light of the described conformational changes reported to occur in the transition between OVA and S-OVA.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Ovalbumin (OVA or gal d 2) is the major egg allergen making up to 54% of the weight of egg white (EW) proteins (Lechevalier, Croguennec, Nau, & Guérin-Dubiard, 2007). It is a phosphoglycoprotein with a molecular mass of 45 kDa (Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981), comprised of 385 amino acids (McCreynolds et al., 1978) and classified as a member of the serine proteinase inhibitor family, although it has no inhibitory activity in its native form (Hunt & Dayhoff, 1980; Stein, Finch, Turnell, McLaughlin & Carrell, 1990).

During the storage of eggs, OVA is converted into a more heat-stable protein, S-ovalbumin (S-OVA) (Smith, 1964; Smith & Back, 1965), with an increased denaturation temperature of 92.5 °C as measured by differential scanning calorimetry (DSC) (Donovan, Mapes, Davis, & Garibaldi, 1975). The transition into an intermediate form between OVA and S-OVA, which has been described in some studies, seems to be necessary for S-OVA formation (Donovan, Mapes, Davis, and Garibaldi, 1975; Hegg, Martens, & Lofqvist, 1979; Ishimaru, Ito, Tanaka, & Matsudomi, 2010). The conformational features of S-OVA are still under research but, so far, it is accepted that the overall structure of S-OVA is quite similar to that of native OVA, except that the amino acids Ser 164, Ser 236, and Ser 320 take the D-configuration and the side chains of Phe 99 and Met

241 undergo conformational changes (Ishimaru et al., 2010; Yamasaki, Takahashi, & Hirose, 2003). An increase of anti parallel β -sheet conformation in detriment of α -helix has also been reported (Kint & Tomimatsu, 1979).

S-OVA represents up to 5% of the content of native OVA in fresh EW, but more than half of OVA is converted to S-OVA by the time the eggs reach the grocery store and, finally, the consumer (Lechevalier et al., 2007). After 6 months of storage at low temperature, the percentage of S-OVA can reach 81% (Vadehra & Nath, 1973). S-OVA can also be formed *in vitro* at alkaline pH and high temperature, for example by incubation at 50 °C for 20 h in 100 mM sodium phosphate at pH 10 (Donovan et al., 1975). The presence of S-OVA in eggs is usually related to a loss of EW functionality, because it induces runny whites with poor congealing capacity (Shitamori, Kojima, & Nakamura, 1984). Most of the work on S-OVA has focused on the quality of stored eggs and related products (Huntington & Stein, 2001), but not on other important topics, such as its allergenicity or digestibility, which, as far as we know, have not been explored as yet.

Resistance to digestion is considered one of the main features that reflect a food allergen's capacity to stimulate a specific immune response (Astwood, Leach, & Fuchs, 1996). The higher structural stability reported for S-OVA may contribute to keep its integrity through the gastro duodenal tract, increasing its ability to sensitise or trigger allergic responses. From this background, the aim of this work was to assess the effect of the transformation of OVA into S-OVA (either by storage or *in vitro*) on its digestibility, estimated by using an *in vitro* model that mimics physiological conditions, as well as on its potential allergenicity, measured in terms of human-IgE binding by inhibition ELISA.

Abbreviations: DD, duodenal digestion; EW, egg white; GD, gastric digestion; OVA, ovalbumin; SGF, simulated gastric fluid; S-OVA, S-ovalbumin.

* Corresponding author. Tel.: +3491 0017 938.

E-mail address: e.molina@csic.es (E. Molina).

2. Material and methods

2.1. Formation of S-OVA *in vitro*

S-OVA samples were prepared from a 50 g/l solution of hen's egg OVA grade VI from Sigma–Aldrich (St. Louis, MO, USA) in milli-Q water, following Smith and Back, 1965 and from EW, obtained from fresh eggs, following Castellano et al., 1996. The pH of the samples was set at 9.9 by drop wise addition of 0.5 M sodium hydroxide. They were heat treated in a water bath at 55 °C for 16 h in 50 ml sealed centrifuge tubes. Then, the samples were cooled down to room temperature (RT) and the pH was adjusted to 4.7 with 0.1 M acetic acid. Denatured proteins were removed by centrifugation at 14,700 g for 10 min and the supernatant was dialysed extensively overnight at 4 °C, using a Spectra/por® Membrane (MWCO 6–8000, Spectra Medical Industries, Inc., Rohini, New Delhi, India). The samples were freeze dried and kept at –20 °C until use. All experiments were carried out in duplicate.

2.2. Formation of S-OVA on storage

Fresh eggs were stored at 4 °C for 6 months. The EW, manually separated from the yolks, were pooled and gently homogenised to avoid foam formation. The pH and weight were checked and the samples were freeze-dried and kept at –20 °C until further analyses. All experiments were carried out in duplicate.

2.3. Differential scanning calorimetry (DSC)

Freeze dried samples were dissolved in 0.05 M Tris-buffer at pH 9.01 to a final concentration of 40 g/l. The denaturation temperature of the samples was determined by DSC (Q1000, TA Instruments Inc., New Castle, DE, USA) by heating from 20 to 120 °C in hermetic aluminium crucibles at a heating rate of 10 °C/min. The denaturation temperatures were determined as the midpoint temperature of the endothermic peak using the Universal Analysis 2000 software (TA Instruments Inc.).

2.4. *In vitro* gastro duodenal digestion

In vitro digestions were carried out in duplicate as previously described (Martos, Contreras, Molina, & López-Fandiño, 2010; Moreno, Mellon, Wickham, Bottrill, & Mills, 2005). Freeze-dried samples were dissolved in simulated gastric fluid (SGF, 35 mM NaCl pH 2), preheated for 15 min at 37 °C and porcine pepsin (EC 3.4.23.1, 3440 units/mg, Sigma–Aldrich) was added at an enzyme/substrate ratio of 1:20 w/w. Aliquots of the gastric digestions were taken at 30 and 60 min. The reaction stopped by raising the pH to 7.0 with 1 M sodium bicarbonate, resulting in a final protein concentration of 5.07 mg/ml.

The simulated duodenal digestion was performed on the 60 min gastric digests at pH 7.0. 1 M calcium chloride, 0.25 M bis-Tris, pH 6.5, and a 0.125 M bile salt mixture, containing equimolar quantities of sodium taurocholate (Sigma–Aldrich) and glycodeoxycholic acid (Sigma–Aldrich), were added. The mixture was heated at 37 °C for 15 min, and the following enzymes dissolved in milli-Q water were added in this order: Corolase PP (AB Enzymes GmbH, Darmstadt, Germany), porcine pancreatic lipase (EC 232-619-9, Sigma–Aldrich), and colipase (EC 259-490-1, Sigma–Aldrich). Aliquots were taken at 30 and 60 min of duodenal digestion and the enzyme activity was stopped by heating at 80 °C for 5 min. The final composition of the mixture was 4.12 mg/ml protein, 6.15 mM of each bile salt, 20.3 mM bis-Tris, 7.6 mM calcium chloride, 28.9 units of lipase/mg and Colipase and Corolase PP enzyme/substrate ratios

were 1:895 w/w and 1:25 w/w, respectively. All the aliquots were kept at –20 °C until further use.

2.5. Reverse phase – high performance liquid chromatography (RP-HPLC)

Aliquots taken during gastro duodenal digestion were dissolved in milli-Q water to a final concentration of 2.5 mg/ml. After filtration through 0.2 µm nitrocellulose filters, the digests were separated by RP-HPLC using a Hi-Pore RP-318 (250 × 4.6 mm) column (Bio-Rad) in a Waters 600 HPLC (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and a 2487 dual wavelength absorbance detector. Elution was performed using 0.37% (v/v) trifluoroacetic acid in milli-Q water, as solvent A, and 0.27% (v/v) trifluoroacetic acid in acetonitrile, as solvent B. Elution was performed as described by Quirós, Chicón, Recio, and López-Fandiño (2007) with slight modifications. In brief, a linear gradient of 0–60% B was applied over 60 min. After 35 min at 60% B, 100% B was reached in 1 min, maintained during 10 min, and finally decreased to 0% in 1 min. The flow rate was maintained at 1 ml/min and peptides were detected by monitoring at 220 nm. Data were processed by using Empower 2 Software (Waters Corporation).

2.6. Human-IgE Binding by Inhibition ELISA

Individual serum samples from children with proven allergy to EW were collected at the Hospital General Universitario Gregorio Marañón. All patients showed specific IgE antibodies towards EW and OVA (Table 1), as estimated by the Immuno-CAP method (Pharmacia Diagnostic, Uppsala, Sweden) and exhibited clinical allergic symptoms. Inhibition ELISA followed Jiménez-Saiz, Martos, Carrillo, López-Fandiño, and Molina (2011) with slight variations. Commercial OVA diluted in 0.01 M phosphate buffer, pH 7.4 (PBS) to 10 µg/ml was used as a coating a coating agent for the analyses of OVA and *in vitro*-formed S-OVA (from OVA or EW), while EW was used as coating agent for the analyses of EW and stored EW. Polyclonal rabbit anti-human IgE (A0094, Dako, Glostrup, Denmark) and polyclonal swine anti-rabbit immunoglobulins labelled with horseradish peroxidase (P0399, Dako) were used diluted 1:1000 and 1:2000 (v/v), respectively, in PBS containing 0.05% Tween 20 (PBST). A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal curve of inhibition dose-response with variable slope, from which the IC₅₀ (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad Prism® package (GraphPad Software Inc., San Diego, CA, USA). The IgE binding capacity was expressed as the percentage of the IC₅₀ of the intact protein: OVA for the analyses of OVA and *in vitro*-formed S-OVA (from OVA or EW) and EW for the analyses of EW and stored EW. Significant differences ($P < 0.05$) were evaluated by one-way analysis of variance followed by post hoc multiple-comparison using Tukey's test.

Table 1

Specific IgE levels (kU/L) towards egg white (EW), ovalbumin (OVA) and ovomucoid (OM) of the sera used in the study.

Patient	IgE levels (kU/L)		
	Egg white	OVA	OM
1	–	62	80
2	100	100	97.1
3	Egg > 100		
4	>100	78.9	69.2
5	Egg > 100		

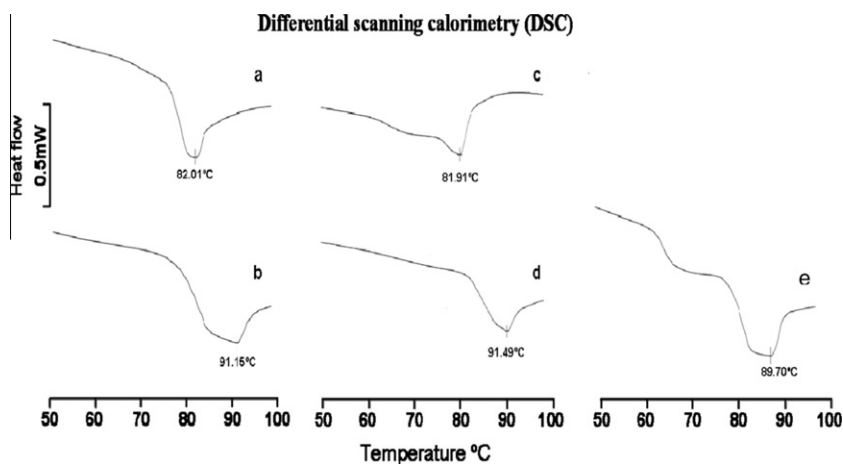


Fig. 1. DSC thermograms with endothermic heat flow curves of commercial OVA (a), fresh egg white (EW) (c), S-OVA formed *in vitro* from commercial OVA (b), S-OVA formed *in vitro* from fresh EW (d), EW after and 6 months of storage at 4 °C (e).

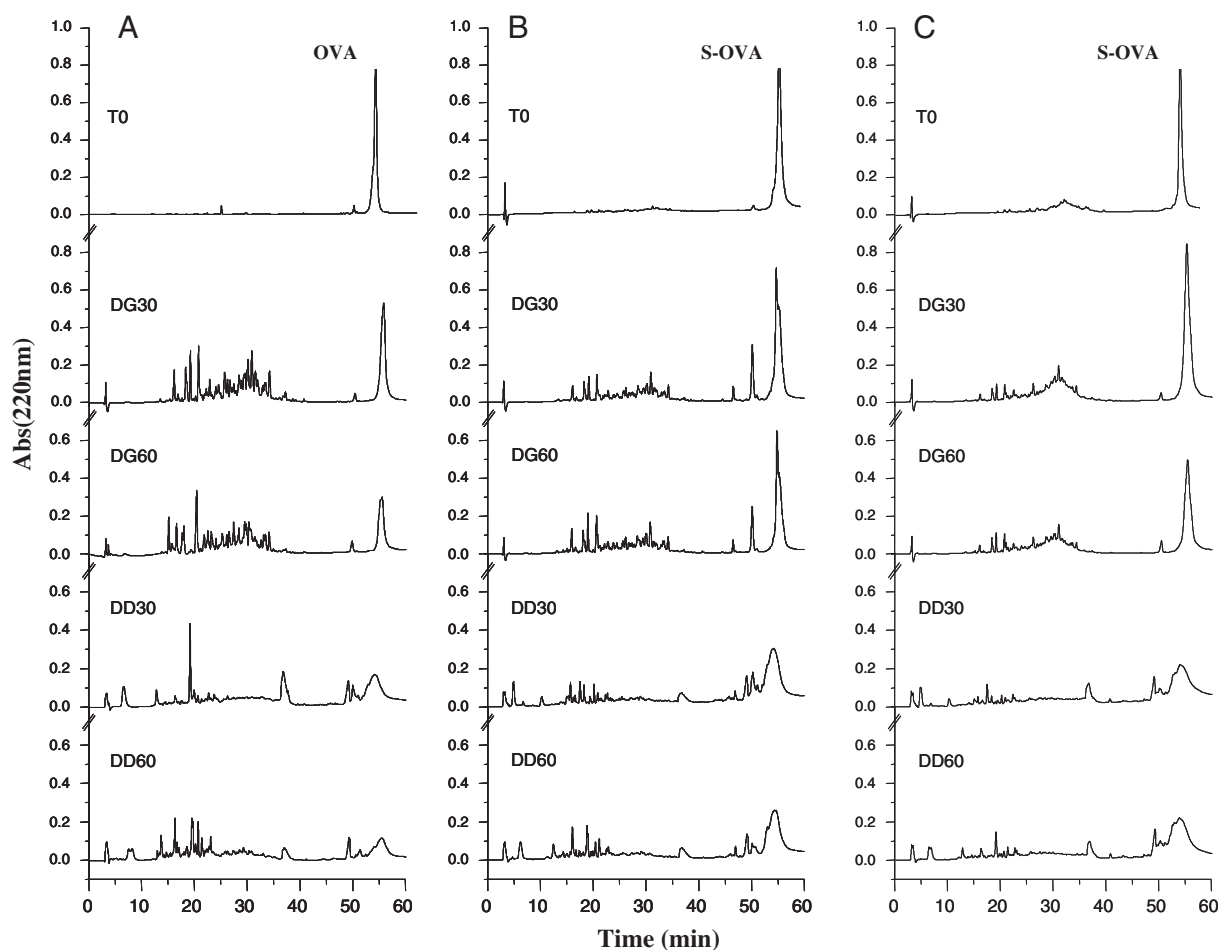


Fig. 2. RP-HPLC patterns corresponding to native OVA (A) and S-OVA subjected to simulated gastric (DG) and duodenal (DD) digestions for 30 and 60 min. S-OVA was formed *in vitro* by heating to 55 °C at high pH either from commercial OVA (B) or from fresh egg white (C).

3. Results and discussion

3.1. S-OVA formation

S-OVA formation was confirmed by DSC analyses (Fig. 1). Native OVA showed a denaturation temperature (T_D) of 82 °C, in

agreement with previous studies (Hammershoj, Larsen, Andersen, & Qvist, 2002), and its *in vitro* incubation under alkaline conditions produced a clear shift in the endothermic peak and a T_D of 90.3 °C, close to the value of 92 °C reported by Donovan et al. (1975) for S-OVA. Similarly, when fresh EW was used as starting material for the *in vitro* conversion, formation of S-OVA was characterised

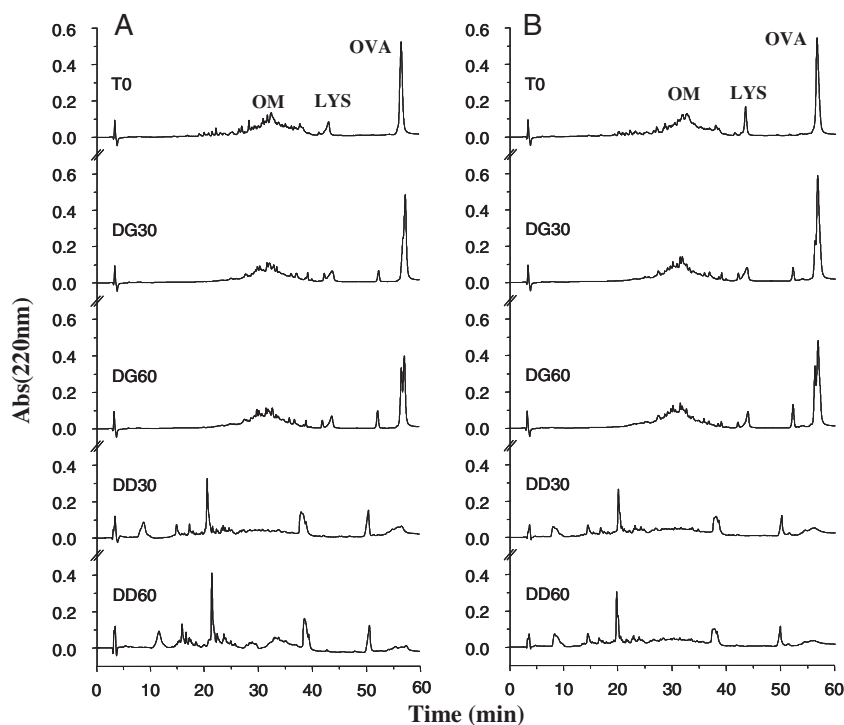


Fig. 3. RP-HPLC patterns corresponding to fresh egg white (EW) (A), and EW stored for 6 months at 4 °C (B), subjected to simulated gastric (DG) and duodenal (DD) digestions for 30 and 60 min. LYS: lysozyme; OM: ovomucoid.

by a marked displacement of the endothermic peak and an increase in T_D from 81.7 to 91.5 °C. The attempts to prepare S-OVA from fresh EW upon storage led to smaller increase in T_D , which was attributed to the low pH attained after the 6-month storage period (9.07). The DSC thermograms suggested that storage at 4 °C for 6 months probably yielded a mixture of OVA, intermediate S-OVA and S-OVA (Fig. 1).

3.2. *In vitro* digestion

OVA and S-OVA were digested by using an experimental model in two steps, which mimics gastric and duodenal physiological conditions (Martos et al., 2010; Moreno et al., 2005). OVA and S-OVA formed from commercial OVA or EW by *in vitro* treatments were indistinguishable by RP-HPLC (Fig. 2) and, in all cases, a large peak of intact protein was still present after pepsin digestion, that partially resisted pancreatic enzymes. The low susceptibility of OVA to proteolysis is in agreement with previous studies (Martos et al., 2010; Takagi, Teshima, Okunuki, & Sawada, 2003). Moreover, S-OVA formed *in vitro* was even more resistant than OVA to the hydrolysis by pepsin, as revealed by a higher amount of intact protein and a lower presence of degradation products after 30 and 60 min of simulated gastric digestion. The enhanced susceptibility to proteolysis of S-OVA with respect to OVA was not so evident after the simulated duodenal digestions. EW stored for 6 months, was also less prone to pepsin action when compared with fresh EW (Fig. 3), although the effect was less pronounced, probably because of the presence of both OVA and S-OVA in the stored sample, as explained above.

From these results, it can be drawn that S-OVA was more resistant to *in vitro* digestion under physiological conditions, possibly as a result of the structural changes associated to the transition of OVA into S-OVA that provide S-OVA with greater stability, compactness and hydrophobicity (Huntington and Stein, 2001).

3.3. Human-IgE binding

The IgE-binding capacity of OVA, S-OVA and their duodenal digests were compared by competitive ELISA using human sera from egg allergic patients (Fig. 4). Even if the individual sera used varied in their specificity towards the egg proteins and their digests, overall, S-OVA formed *in vitro* showed a much lower reactivity against IgE than native OVA (Fig. 4A and B). This lower IgE-binding could be explained because of the structural changes undergone by S-OVA, in particular, those involving the amino acids Phe 99, Ser 164 and Ser 320, all of which form part of previously reported IgE epitopes recognised by allergic subjects, such as the large CNBr-fragments 41–172 and 301–385. These fragments, according to Kahlert, Petersen, Becker & Schlaak (1992), react with patients' IgE. Mine and Rupa (2003) determined the entire mapping of the IgE-binding epitopes in the primary sequence of OVA, finding five distinct regions mainly composed of hydrophobic residues, one of which (95–102) included Phe 99. Furthermore, according to Yamasaki et al. (2003), the conformational change in Phe 99 side chain in S-OVA induces a decrease in the solvent accessibility of the surrounding residues which include Phe 378, a residue that forms part of an allergenic epitope of OVA which is exposed at the surface of the protein (residues 347–385, Honma et al., 1996). On the other hand, the observed 2–5% loss of α -helix and the 3–4% increase in the β -sheet content in S-OVA, as well as its higher binding capacity against a hydrophobic fluorescent dye, are assumed to result from a partial denaturation during the alkaline treatment (Yamasaki et al., 2003) that could have contributed to the rupture of conformational epitopes with the subsequent reduction in IgE-binding. Regarding EW stored at 4 °C, competitive ELISA showed that it exhibited an IgE binding closer to than that of native EW, with some differences depending on the individual serum used (Fig. 4C). This could be attributed to the observation that native OVA was only partially converted into S-OVA and to the presence of other immunogenic egg-white proteins in the stored

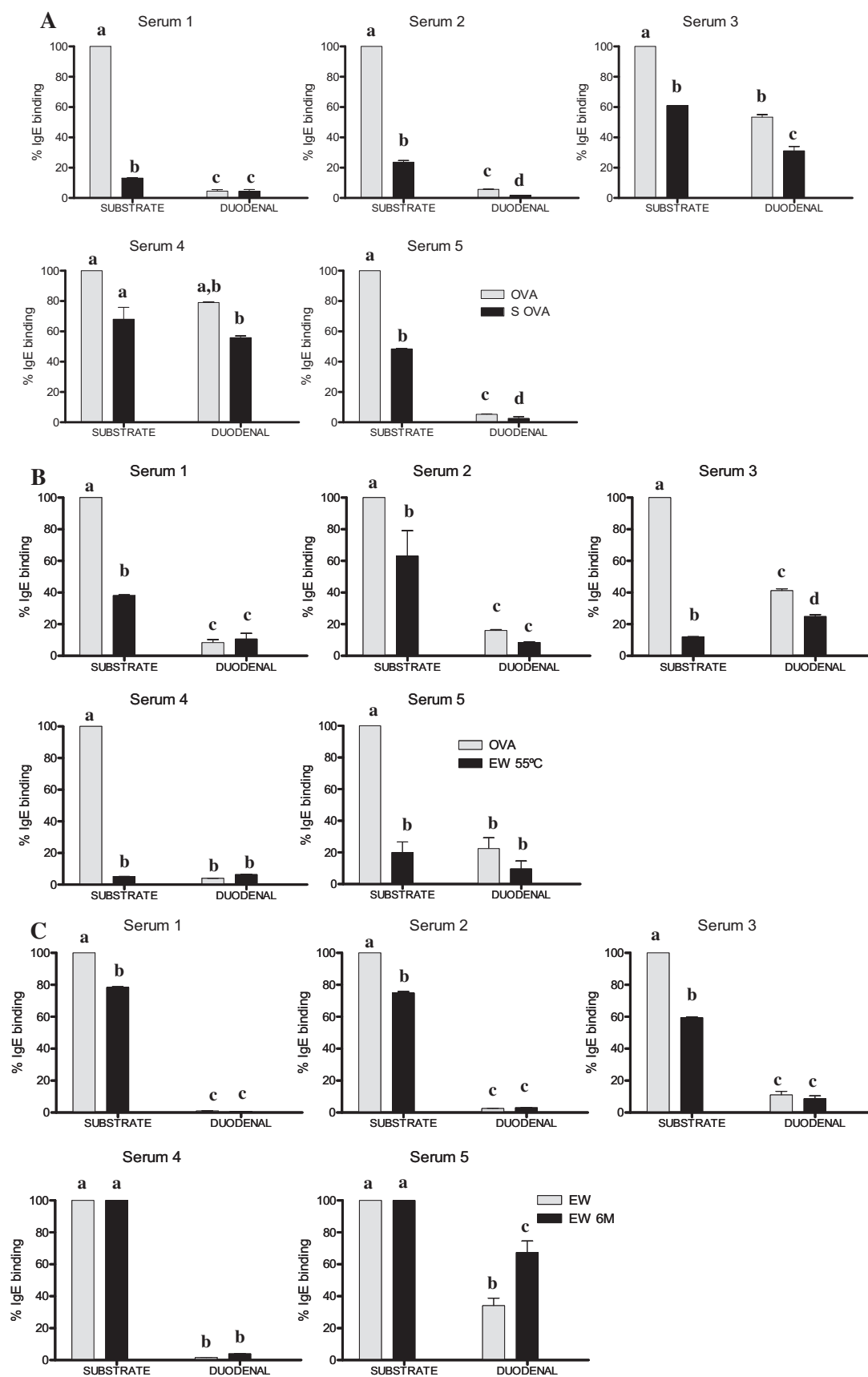


Fig. 4. Inhibition ELISA response against human IgE of S-OVA formed *in vitro* from commercial OVA (A), from egg white (EW) (B) and formed upon storage for 6 months at 4 °C from EW (C). The IgE-binding capacities were expressed as the percentage of the IC50 of the intact protein: OVA for the analyses of OVA and *in vitro*-formed S-OVA (from OVA or EW) (A and B) and EW for the analyses of EW and stored EW (C). Bars correspond to the 95% confidence intervals and different letters indicate significant differences ($P < 0.05$).

EW (such as ovomucoid and lysozyme) that could also contribute to its IgE-binding.

Despite the lower IgE-binding of S-OVA and its higher stability towards digestion, the duodenal digests of OVA and S-OVA formed *in vitro* showed a reduced but comparable reactivity towards IgE. The same happened in the case of fresh and stored EW. This could be explained by the similarity in their duodenal digestion patterns (Figs. 2 and 3) that point to a similar contribution of peptides containing intact binding sites recognisable by serum IgE, which are the compounds most probably responsible for the low, but detectable, ELISA response. It is therefore, likely that the transformation of OVA to S-OVA does not affect its ability to sensitise or trigger allergic reactions at the duodenal level.

4. Conclusions

S-OVA, formed from OVA either *in vitro* or upon storage, showed less capacity to bind human IgE and higher resistance to proteolysis, particularly to pepsin. Despite these differences, which can be attributed to the conformational changes reported to occur in the transition between OVA and S-OVA, the *in vitro* duodenal digests of both protein forms were similar in terms of IgE binding, which suggests that the transformation of OVA to S-OVA would not affect its allergenic potential at the duodenal level.

Acknowledgements

Jiménez-Saiz and Pineda-Vadillo are thankful to MICINN and CSIC for financial support. This work received financial support from the projects AGL2008-01740, CONSOLIDER CSD2007-00063 FUN-C-FOOD (Spanish Ministry of Education and Science) and S-505/AGR-0153 (Madrid Autonomous Community). We thank Mr. Negueruela and Ms. Bravo the supply of fresh eggs.

The authors have declared no conflict of financial/commercial interest

References

- Astwood, J., Leach, J. N., & Fuchs, R. L. (1996). Stability of food allergens to digestion *in vitro*. *Nature Biotechnology*, 14, 1269–1273.
- Castellano, A. C., Barteri, M., Bianconi, A., Bruni, F., DellaLong, S., & Paolinelli, C. (1996). Conformational changes involved in the switch from ovalbumin to S-ovalbumin. *Zeitschrift für Naturforschung C-A Journal of Biosciences*, 51, 379–385.
- Donovan, J. W., Mapes, C. J., Davis, J. G., & Garibaldi, J. A. (1975). Differential scanning calorimetric study of stability of egg-white to heat denaturation. *Journal of the Science of Food and Agriculture*, 26, 73–83.
- Hammershoj, M., Larsen, L. B., Andersen, A. B., & Qvist, K. B. (2002). Storage of shell eggs influences the albumen gelling properties. *Lebensmittel-Wissenschaft Und Technologie-Food Science and Technology*, 35, 62–69.
- Hegg, P. O., Martens, H., & Lofqvist, B. (1979). Effects of Ph and neutral salts on the formation and quality of thermal aggregates of ovalbumin - study on thermal aggregation and denaturation. *Journal of the Science of Food and Agriculture*, 30, 981–993.
- Honma, K., Kohno, Y., Saito, K., Shimojo, N., Horiuchi, T., Hayashi, H., Suzuki, N., Hosoya, T., Tsunoo, H., & Niimi, H. (1996). Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: Inhibition of basophil histamine release by haptenic ovalbumin peptide. *Clinical and Experimental Immunology*, 103, 446–453.
- Hunt, L. T., & Dayhoff, M. O. (1980). A surprising new protein superfamily containing ovalbumin, anti-thrombin-III, and alpha1-proteinase inhibitor. *Biochemical and Biophysical Research Communications*, 95, 864–871.
- Huntington, J. A., & Stein, P. E. (2001). Structure and properties of ovalbumin. *Journal of Chromatography B: Biomedical Science and Applications*, 756, 189–198.
- Ishimaru, T., Ito, K., Tanaka, M., & Matsudomi, N. (2010). Thermostabilization of ovalbumin by alkaline treatment: Examination of the possible roles of D-serine residues. *Protein Science*, 19, 1205–1212.
- Jiménez-Saiz, R., Martos, G., Carrillo, W., López-Fandiño, R., & Molina, E. (2011). Susceptibility of lysozyme to in-vitro digestion and immunoreactivity of its digests. *Food Chemistry*, 127, 1719–1726.
- Kahlert, H., Pertersen, A., Becker, W. M., & Schlaak, M. (1992). Epitope analysis of the allergen ovalbumin (Gal-D-II) with monoclonal-antibodies and patients IgE. *Molecular Immunology*, 29, 1191–1201.
- Kint, S., & Tomimatsu, Y. (1979). Raman difference spectroscopic investigation of ovalbumin and S-ovalbumin. *Biopolymers*, 18, 1073–1079.
- Lechevalier, V., Croguennec, T., Nau, F., & Guérin-Dubiard, C. (2007). Ovalbumin and gene-related proteins. In R. Huopalahti, R. López-Fandiño, M. Anton, & R. Schade (Eds.), *Bioactive egg compounds* (pp. 145–158). Berlin Heidelberg: Springer-Verlag.
- Martos, G., Contreras, P., Molina, E., & López-Fandiño, R. (2010). Egg white ovalbumin digestion mimicking physiological conditions. *Journal of Agricultural and Food Chemistry*, 58, 5640–5648.
- McCreynolds, L., Omalley, B. W., Nisbet, A. D., Fothergill, J. E., Givol, D., Fields, S., et al. (1978). Sequence of chicken ovalbumin messenger-Rna. *Nature*, 273, 723–728.
- Mine, Y., & Rupa, P. (2003). Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken ovalbumin. *Protein Engineering*, 16, 747–752.
- Moreno, F., Mellon, F. A., Wickham, M. S. J., Bottrill, A. R., & Mills, E. N. C. (2005). Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant *in vitro* gastrointestinal digestion. *The FEBS Journal*, 272, 341–352.
- Nisbet, A. D., Saundry, R. H., Moir, A. J. G., Fothergill, L. A., & Fothergill, J. E. (1981). The complete amino-acid-sequence of hen ovalbumin. *European Journal of Biochemistry*, 115, 335–345.
- Quirós, A., Chicón, R., Recio, I., & López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry*, 104, 1734–1739.
- Shitamori, S., Kojima, E., & Nakamura, R. (1984). Studies on ovalbumin-S-ovalbumin Transformation. 4. Changes in the Heat-Induced Gelling Properties of ovalbumin During Its Conversion to S-Ovalbumin. *Agricultural and Biological Chemistry*, 48, 1539–1544.
- Smith, M. B. (1964). Studies on ovalbumin. 1. Denaturation by heat + heterogeneity of ovalbumin. *Australian Journal of Biological Sciences*, 17, 261–270.
- Smith, M. B., & Back, J. F. (1965). Studies on ovalbumin. 2. Formation and properties of S-ovalbumin a more stable form of ovalbumin. *Australian Journal of Biological Sciences*, 18, 365.
- Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J., & Carrell, R. W. (1990). Crystal-Structure of ovalbumin as a model for the reactive center of serpins. *Nature*, 347, 99–102.
- Takagi, K., Teshima, R., Okunuki, H., & Sawada, J. (2003). Comparative study of *in vitro* digestibility of food proteins and effect of preheating on the digestion. *Biological & Pharmaceutical Bulletin*, 26, 969–973.
- Vadehra, D. V., & Nath, K. R. (1973). Eggs as a source of protein. *CRC Critical Reviews in Food Science and Nutrition*, 4, 193–309.
- Yamasaki, M., Takahashi, N., & Hirose, M. (2003). Crystal structure of S-ovalbumin as a non-loop-inserted thermostabilized serpin form. *Journal of Biological Chemistry*, 278, 35524–35530.