



Analytical Methods

Interactions of epigallo-catechin 3-gallate and ovalbumin, the major allergen of egg white



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ABSTRACT

Polyphenols, the potent plant secondary metabolites, have beneficial effects on human health, but the mechanism(s) by which these effects are exerted is not well understood. Here, we present the detailed analysis of the interactions between the major green tea catechin, epigallo-catechin 3-gallate (EGCG), and the major dietary protein and allergen, ovalbumin (OVA). We show that EGCG binds to the pocket that partly overlaps with the previously identified IgE-binding region in OVA, and that this interaction induces structural changes in the allergen. Moreover, our *ex vivo* studies reveal that OVA binds IgE and stimulates degranulation of basophils, and that its uptake by monocytes proceeds at a slower rate in the presence of EGCG. This study provides further evidence in support of the proposed mechanism by which EGCG interactions with the food allergens contribute to its diverse biological activities and may impair antigen uptake by antigen-presenting cells.

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1. Introduction

Egg is one of the most prominent sources of important nutrients in human diet. Ovalbumin (OVA), the most prevalent protein in hen egg white (~60–65% of total protein content) is a glycoprotein, which consists of 385 amino acids, and represents the major allergen from avian egg white that causes IgE-mediated food allergic reactions particularly in children; the prevalence of OVA allergy is estimated between 1.6% and 3.2% among children and 0.6% among adults (Sampson, 2004). Based on sequence homology and its three-dimensional structure, OVA has been classified as a member of the large superfamily of SERine Protease Inhibitors (serpin). Serpins are single-domain proteins with a distinct fold composed of three β -sheets (A–C), which form the core of the structure, and 8–9 α -helices distributed on the surface of the molecule. On top of the central β -sheet A there is a 15–20 residue long

solvent-exposed loop, which is known as the reactive center loop (RCL) that serves as a bait for the target protease. In contrast to inhibitory serpins, OVA does not show protease inhibitor activity and is thus referred to as a non-inhibitory serpin. Moreover, while other non-inhibitory serpins, such as maspin and pigment epithelium derived factor (PEDF), have defined biological roles ranging from regulating angiogenesis to tumor suppression, the true biological role of OVA, apart from possibly being used as storage protein, is far from clear (Huntington & Stein, 2001). The involvement of OVA in allergic reactions and its ability to interact with small physiologically active molecules, such as polyphenols and purine alkaloids, warrants thorough analysis (Wang et al., 2013).

The majority of studies on the interactions between polyphenols and proteins have yielded binding constants, putative polyphenol-binding sites, the character of forces important for recognition, and effects of the complex formation on the stability and antioxidant activity of polyphenols (Kanakakis et al., 2011).

It has recently been shown that consumption of polyphenol-enriched apple extract reduced symptoms of OVA food allergy in a mouse model (Zuercher, Holvoet, Weiss, & Mercenier, 2010). Apple polyphenols were also able to form complexes with OVA, and to reduce specific antibody binding and release of serotonin from basophils. Proposed mechanisms of anti-allergic action of

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apple polyphenols were related, at least in part, to polyphenol–OVA interactions, which presumably reduced allergenicity of the allergen by epitope modification. Gruber et al. reported similar results with the major cherry allergen Pru av 1 (Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). In particular, it has been shown that the interaction of Pru av 1 with various polyphenols, including epicatechin, gallic acid and quercetin, led to permanent modification of the tertiary structure of the allergen, which resulted in a strong reduction in its IgE-binding capacity. Recently, similar findings demonstrated reduced allergenicity of peanuts after interaction with polyphenols (Si-Yin Chung, 2009).

(–)-Epigallo-catechin 3-gallate (EGCG) is the major active principle of green tea, which exerts beneficial effects on human health, including hypersensitivity disorders (Melgarejo, Medina, Sanchez-Jimenez, & Urdiales, 2010). Frequently ingested in combination with protein foods, this very abundant green tea polyphenol exerts various biological activities that may affect human health and modulate immune reactivity to dietary proteins. Several studies demonstrated non-covalent interactions between EGCG and major dairy proteins, β -lactoglobulin and casein (Hasni et al., 2011). Binding of EGCG to proteins leads to conformational changes in the protein (Kanakakis et al., 2011), and affects proteolysis by pepsin (Tagliazucchi, Verzelloni, & Conte, 2005; Tantoush et al., 2013). Protein conformation, digestibility and aggregation are important for biological activities of dietary proteins that elicit hypersensitivity reactions in humans, such as IgE-binding capacity, uptake by antigen-presenting cells, activation of effector cells in allergy, and sensitising potential of food allergens (Visser et al., 2011).

Here, we present the detailed analysis of the non-covalent interaction between EGCG and the major protein from hen egg, ovalbumin, and the effect of the OVA/EGCG complex formation on the allergen structure, allergen-IgE antibody recognition, degranulation of effector cells in allergy (basophils) and protein uptake by monocytes.

2. Material and methods

The study was approved by the local ethics committee of the University Children's Hospital in Belgrade. Patients (allergic and non-allergic) gave informed consent.

2.1. Materials

OVA was purified according to a previously published method, with minor modifications (Omana, Wang, & Wu, 2010). Protein purity was assessed by electrophoresis. (–)-Epigallo-catechin 3-gallate (EGCG), RPMI-1640 medium, DMSO, mouse anti-human IgE labelled with alkaline phosphatase, Ponceau S, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were also from Sigma Aldrich (St. Louis, MO, USA). Antibody anti-CD63 was purchased from BD Bioscience (New Jersey, USA) and anti-ENPP3 was purchased from Abcam (Cambridge, UK). Deionised water (DW) used in the experiments was purified in a Milli-Q system (Millipore, Molsheim, France).

2.2. Electrophoresis and immunoblotting

SDS–PAGE was carried out using Hoefer scientific instrumentation (Amersham Biosciences) with a discontinuous buffer system on 14% polyacrylamide gel. Samples were analysed under reducing conditions. Protein transfer was done on NC membrane (Roti-NC 0.2 μ m) at 1.5 mA/cm² of gel for 45 min. After transfer membrane was stained using Ponceau S.

2.3. Fluorescence spectroscopy

Fluorescence spectra were recorded on FluoroMax-4 spectrofluorimeter (Horiba Scientific, Kyoto, Japan). Experiments were carried out at +22 °C in a 3.5 mL quartz cuvette, 20 mmol L^{–1} phosphate buffer pH 7.2 was used. Protein solution (25 μ g mL^{–1}) was titrated with 11 $\times$ 1 μ L and 4 $\times$ 1, 25 μ L of EGCG (2.5 mg mL^{–1}) at pH 7.2. After the addition of each aliquot, a fluorescent spectrum was recorded immediately at: λ excitation 280 nm, λ emission 290–500 nm. Between each measurement, the cell was washed three times with deionised water. A blank sample containing the buffer and appropriate polyphenol concentration was measured. The resulting blank spectrum was automatically subtracted from the emission spectrum of the corresponding solution. All experiments were performed in triplicate and the averaged data obtained from the binding studies were used for the calculations of the binding parameters. Spectra were further analysed in OriginPro 8 software package (Northampton, MA, USA). Fluorescence spectra analysis by Stern–Volmer equation (Liang, Tajmir-Riahi, & Subirade, 2008) is described in Supporting On-line Material (SOM).

2.4. Isothermal titration calorimetry (ITC)

All ITC measurements were taken on MicroCal iTC200 (GE Lifesciences, USA) and experimental details are shown in SOM.

2.5. Circular dichroism and secondary structure calculation

CD spectra of protein/EGCG samples were recorded on a JASCO J-815 spectropolarimeter (JASCO, Japan) at 25 °C in 1 nm steps at a rate of 50 nm/min over the wavelength range 185–260 nm using a quartz cell with a path length of 0.01 cm in nitrogen atmosphere. Protein concentration was 23 μ mol L^{–1} while EGCG concentration was 23 μ mol L^{–1} and 235 μ mol L^{–1}. Samples were prepared in 20 mmol L^{–1} sodium-phosphate pH 7.2. Each spectrum was acquired four times, results were averaged and expressed as residue-average molar ellipticity, as described previously (Stanic-Vucinic et al., 2012). To determine the percentage of the secondary structure content, the CONTIN software and the SP29 protein set provided in CDPro software package were used. Statistical analysis was performed using one way ANOVA followed by Dunnett test.

2.6. Docking analysis and structural modeling of the OVA/EGCG binary complex

The crystal structure of uncleaved chicken OVA was extracted from a protein data bank (PDB ID: 1OVA) (Stein, Leslie, Finch, & Carrell, 1991). Docking analysis and structural modeling of the OVA/EGCG binary complex are described in SOM.

2.7. Basophil activation assay

Patients were selected on the basis of positive skin prick testing to eggs, a documented clinical history of food allergy, and positive *in vitro* testing to egg white: IgE levels assessed to egg white with ImmunoCAP[®] 100 system using the ImmunoCAP[®] code f1 (Pharmacia Diagnostics, Uppsala, Sweden) above 0.35 kAU L^{–1}. Basophil activation assay was performed as described previously (Boumiza, Debard, & Monneret, 2005) and experimental details are shown in SOM.

2.8. Western blot inhibition of IgE binding

Membrane was blocked with 1% BSA in Tris buffer saline (TBS), pH 7.4 containing 0.1% of Tween 20 (TTBS) overnight at +4 °C. Membranes were incubated with a pool of 8 patients' sera, total dilution of serum 2.5 $\times$ (patients with proven egg white allergy,

average 27.2 kAU L^{-1}) for 2 h at $+25^\circ\text{C}$ in the absence (positive control), or in the presence of inhibitor (EGCG) in different concentrations: 1, 5, 10, 15 and $100 \mu\text{mol L}^{-1}$. Serum with no proven allergy was used as a negative control. Detection was performed with mouse-anti-human IgE monoclonal antibody (2000 times diluted in TTBS +0.1% BSA) conjugated to alkaline phosphatase for 1 h at RT. The binding patterns were visualised with a substrate solution consisting of 1.5 mg BCIP and 3 mg NBT in 10 mL of 100 mmol L^{-1} Tris buffer, containing 150 mmol L^{-1} NaCl, and 5 mmol L^{-1} MgCl_2 , pH 9.6.

2.9. Uptake of protein–polyphenol complexes by monocytes

2.9.1. FITC labelling of protein

Ovalbumin was conjugated to FITC isomer I (Sigma–Aldrich, Taufkirchen, Germany) following the manufacturer's instructions as described in SOM.

2.9.2. Protein uptake by monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of three healthy adult donors by Ficoll density gradient separation (Histopaque, Sigma–Aldrich, Taufkirchen, Germany). Monocytes were isolated from PBMCs by immunomagnetic cell sorting following the manufacturer's instructions (MACS; Miltenyi–Biotec, Bergisch–Gladbach, Germany) resulting in $>95\%$ CD14+ cells. To access protein uptake, FITC-labelled OVA ($1.1 \mu\text{mol L}^{-1}$) with or without the addition of EGCG ($1.1 \mu\text{mol L}^{-1}$, $11 \mu\text{mol L}^{-1}$) was incubated with 50×10^5 freshly isolated monocytes in 200 μL of cell culturing medium (RPMI 1640 medium supplemented with 10% foetal calf serum, 0.1% gentamicin, 100 U mL^{-1} penicillin, and $100 \mu\text{g mL}^{-1}$ of streptomycin). Uptake proceeded for 0, 0.5, 1, 3 h at 37°C in a humidified atmosphere with 5% CO_2 . Mean fluorescence intensity (MFI) of FITC-positive monocytes was measured by flow cytometry using FACS Calibur (BD Biosciences, USA). As a control, monocytes were incubated with FITC-OVA ($1.1 \mu\text{mol L}^{-1}$) for the same periods of time at $+4^\circ\text{C}$.

GraphPad software was used to analyse data and draw graphs. Graph of MFI vs. time was linearised by dividing measured MFIs with $\text{MFI}_{t=0}$ measured at time point 0. Slopes of regression lines were used as a measure of the uptake rate. To test whether slopes were statistically different, we calculated two-tailed *P* values using GraphPad built in statistical method, which is equivalent to an Analysis of Covariance (ANCOVA).

3. Results

3.1. EGCG binds to a major food allergen, ovalbumin, in solution

It has previously been suggested that plant polyphenols specifically interact with food allergens and that such interactions might modulate immune response in humans (Singh, Holvoet, & Mercenier, 2011; Zuercher et al., 2010). These findings also suggested that polyphenols could be employed in prevention and treatment of allergic diseases. We hypothesised that EGCG might bind to the major dietary protein and food allergen from egg white, ovalbumin. To establish the strength and nature of the putative OVA/EGCG interactions in solution, we employed spectrofluorimetry and isothermal titration calorimetry (ITC).

The fluorescence spectrum of OVA is dominated by Trp fluorescence (Fig. 1A). There are three Trp residues in OVA that are positioned differently in the structure: Trp 160 is solvent exposed, Trp 194 is buried within the hydrophobic pocket, and Trp 275 is partially exposed. Addition of increasing amounts of EGCG causes quenching of OVA fluorescence between 290 and 450 nm (Fig. 1A). Also, a red shift in the emission maximum of OVA, in

the presence of EGCG was observed (Fig. 1A). These findings suggest in the presence of EGCG, the OVA fluorophores become more accessible to the solvent, which presumably increases the propensity of the Trp side chain to form H-bonds.

The quenching of Trp emission at 340 nm was used to estimate the strength of the interaction between OVA and EGCG by applying Stern–Volmer equation and analysis. The intensity of quenching, presented as a linear ratio of F_0/F versus concentration of the quencher, gives the Stern–Volmer quenching constant. As indicated by the linear Stern–Volmer plot (Fig. S1), EGCG quenches OVA fluorescence by a mechanism that allowed determination of quenching rate constants and analysis of the binding affinities. By applying the Stern–Volmer equation, the Stern–Volmer quenching constant (K_{SV}) and the fraction of the accessible fluorophore sites (f_a) (Fig. 1B) were determined to be $3.9 \times 10^4 \text{ L mol}^{-1}$ and 0.70, respectively (SOM Table S1). Assuming the half-life of Trp in OVA is 10 ns (Wang et al., 2013), the bimolecular quenching constant obtained was higher than the rate of the diffusion-limited quenching (the maximum value possible of k_q for diffusion-limited quenching in water is $\sim 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ (Eva Gudgin & Ware, 1981)). Thus, we conclude that a stable OVA/EGCG complex is formed in solution and EGCG quenches OVA fluorescence via a static mechanism, which allowed application of the double-logarithmic equation for determination of the association constant and OVA/EGCG complex stoichiometry. The K_d value for OVA/EGCG interaction, as estimated by double-logarithmic equation, was $1.2 \times 10^4 \text{ L mol}^{-1}$ ($K_d = 83 \mu\text{M}$) with the stoichiometry of 0.94 EGCG molecules bound per OVA (SOM Table S1).

The binding between EGCG and OVA was also supported by ITC experiments (Fig. 1C). The ITC thermogram showed the OVA/EGCG complex formation was accompanied by an exothermic enthalpy change. Because the ITC data could not be interpreted with a single-site binding model, it is plausible that OVA binds multiple ligand molecules at high EGCG concentrations (Fig. 1C).

In conclusion, our results suggest that EGCG binds to OVA and it forms a stable complex with this allergen in solution.

3.2. The binding of EGCG to OVA causes a change in the secondary structure content in OVA

Previous studies have shown that the binding of polyphenols affects the structure of dietary proteins (Kanakakis et al., 2011; Yuksel, Avci, & Erdem, 2010). It has been demonstrated that the binding of EGCG to β -lactoglobulin (BLG) increases the β -sheet content in the protein at the expense of the random coil content suggesting that a conformational change and stabilization of the protein structure occurred in the presence of EGCG (Kanakakis et al., 2011). To establish whether EGCG affects the global structure and the secondary structure content of OVA, we utilised the CD spectroscopy, a non-invasive method amenable for detecting subtle changes in the protein secondary structure.

Our measurements show that the addition of EGCG caused an increase in the OVA β -sheet structure content. The effect was present in a 1:1 M ratio and persisted at higher EGCG concentrations (Fig. 1D). Furthermore, the β -sheet content increase occurs at EGCG concentrations that were around or above the observed K_d (Table 1). This increase is presumably a consequence of the reduction in random coil content (Fig. 1D and Table 2). Thus, we conclude binding of EGCG affects the secondary structure content of OVA through a conformational change.

3.3. Identification of the putative EGCG-binding site in OVA by molecular docking

Molecular docking simulations and theoretical calculations suggested several potential EGCG-binding sites in OVA. Our analysis

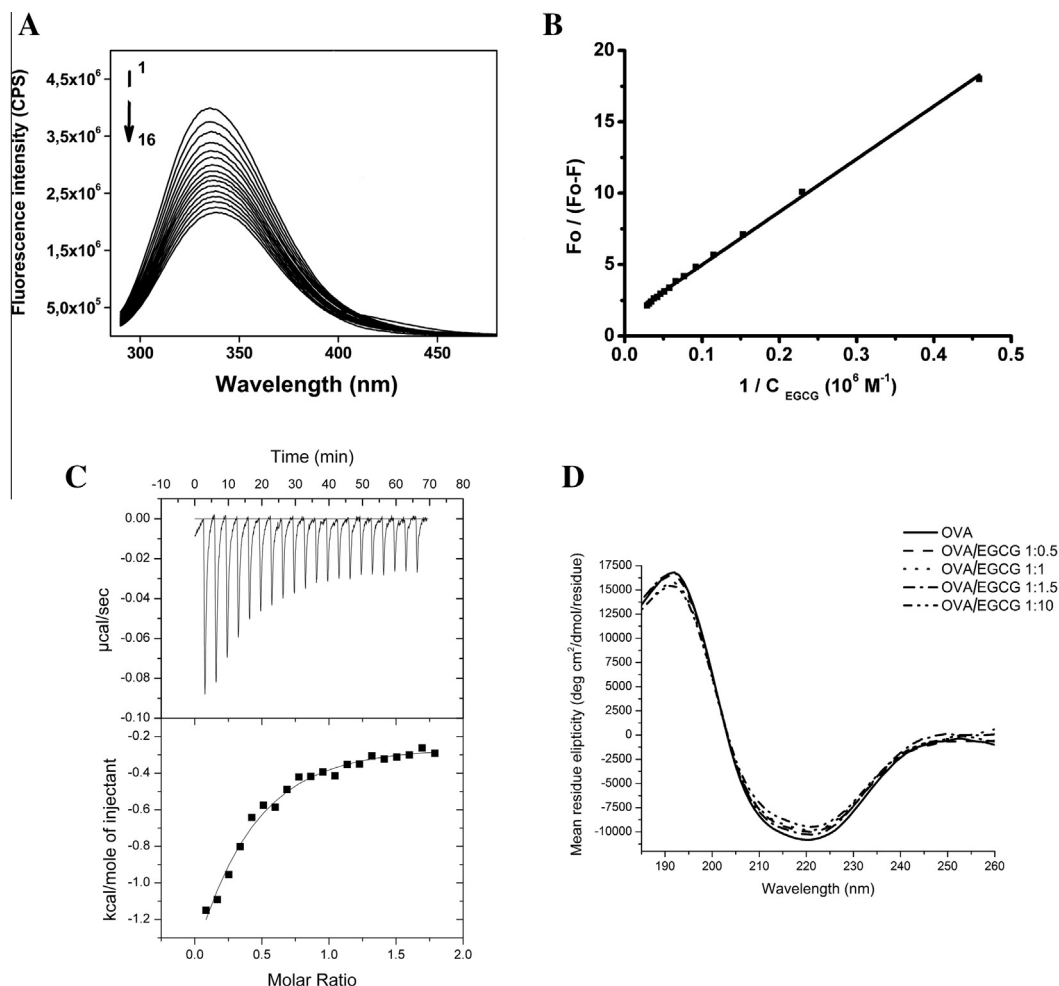


Fig. 1. The effect of EGCG on the OVA fluorescence and CD spectra suggests that EGCG binds to OVA. (A) The fluorophore quenching in OVA by increasing amounts of EGCG (1–16: 0–34.7 μ M). The arrow indicates the trend of the fluorescence change in OVA upon addition of EGCG as well as the trend of the EGCG concentration increase in the experiment. (B) A Stern–Volmer plot for the OVA/EGCG complex. (C) The ITC thermogram reveals that the binding of EGCG to OVA is associated with the exothermic enthalpy change and that OVA might bind multiple ligand molecules at higher EGCG concentrations. (D) EGCG induces changes in the CD spectra of OVA. The CD spectra were recorded in using OVA alone (—) and OVA in the presence of increasing amounts of EGCG. The final OVA/EGCG molar ratios were: 1:0.5 (---), 1:1 (···), 1:1.5 (— · —) and 1:10 (— · — · —).

Table 1

The secondary structure content in OVA determined based on the CD measurements.

OVA/EGCG molar ratio	α -helix	β -strand	Turn	Random
0	29.40	19.10	20.40	31.10
0.5	30.50	21.20	19.70	28.60
1	29.30	25.55	20.05	25.10
1.5	30.90	20.35	19.35	29.40
10	30.05	28.65	18.00	23.30

Table 2

EGCG decreases the rate of OVA uptake by human monocytes.

No.	OVA	OVA/EGCG 1:1	OVA/EGCG 1:10
1 ^a	0.9 $\pm$ 0.1	0.58 $\pm$ 0.09	0.46 $\pm$ 0.09
2 ^b	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1	0.24 $\pm$ 0.06
3 ^c	0.70 $\pm$ 0.07	0.3 $\pm$ 0.2	0.66 $\pm$ 0.05

The uptake curves were linearised by plotting $MFI/MFI_{t=0}$. Curves were compared and slope values were used as a measure of the uptake rate. Table contains slope values ($\pm$ st. error) of three independent experiments with monocytes isolated from three blood donors.

^a Differences between the slopes are significant with $P < 0.0001$.

^b Differences between the slopes are significant with $P = 0.006$.

^c Differences between the slopes are significant with $P = 0.01$.

revealed 12 distinct ligand structures bound to OVA, four of which had binding energies above 32.0 kJ mol⁻¹. Based on the predicted binding energy and the nature of the putative interactions, the most probable binding site is situated on top of helix E (hE) (Fig. 2). In fact, the top 8 structures out of the 12 were found to bind in this particular pocket with slightly different orientations and binding energies. The top hit binds to OVA with -37.6 kJ mol⁻¹, while the next on the list binds with -32.6 kJ mol⁻¹. The remaining four structures bind elsewhere on the protein surface with lower binding energies (-29.3 to -32.2 kJ mol⁻¹). The nature of the interactions at these sites suggests these are of secondary importance and of low affinity. Thus, we focused on the high-affinity interaction between EGCG and OVA only.

The high-affinity site is composed of residues from several structural elements. The floor is composed of side chains from hB, whereas the walls are built of residues from hD, strand 2A (s2A), and a loop connecting hE and s1A (Fig. 2A and C). The trihydroxybenzoate and trihydroxyphenyl rings of EGCG are stacked and on top of the hE-sA1 loop pointing towards s2A and hF, whereas the dihydrobenzopyran ring points in the opposite direction, towards hD (Fig. 2B). The carbonyl oxygen from the trihydroxybenzoate ring interacts with the side-chain O γ from Thr104 (the numbering from the PDB ID 1OVA is used throughout the text). This part of the ligand is further stabilised by H-bonds between its hydroxyl groups and the guanidino group of Arg139,

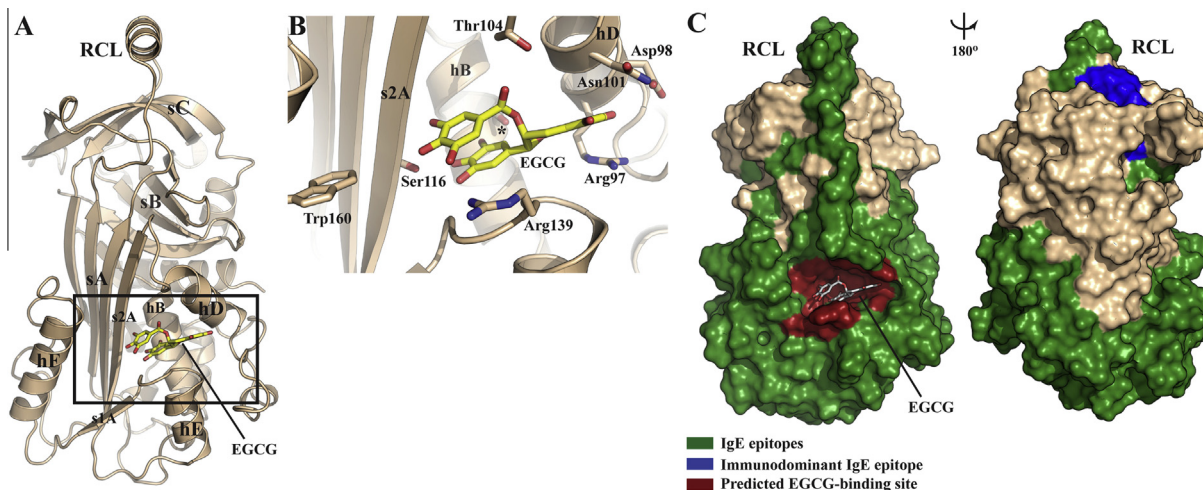


Fig. 2. The molecular docking analysis reveals the putative EGCG-binding site in OVA. (A) The strongest EGCG-binding site is located in the crevice enclosed by hB, hD, hE, s2A, and hE-s1A loop. The box designates the area shown in detail under B. The major structural elements in OVA (beige) are labelled. EGCG is in gold sticks. (B) The close-up view of the mode of EGCG binding. The secondary structure elements constituting the EGCG-binding pocket are highlighted; the amino-acid residues that are in close contact with EGCG and Trp160 are shown as beige sticks; the carbonyl oxygen of Ser59 is labelled with an asterisk. The view is the same as under A. (C) A surface representation diagram of the putative OVA/EGCG complex with the main binding sites highlighted. The view on the left is the same orientation as in A, whereas the view on the right is rotated 180° counterclockwise around the vertical axis. The surface covering main IgE epitopes is green, the immunodominant IgE epitope is blue, the putative EGCG-binding site is dark red, and EGCG is in grey sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the backbone NH group of Ser116 (Fig. 2B). The trihydroxyphenyl ring, which is stacked below the trihydroxybenzoate ring, is held in place by H-bonds formed between its OH groups and the γ atom of Ser59, and the carbonyl oxygens of Ser116 and Arg139 (Fig. 2B). These interactions bring the trihydroxybenzoate and trihydroxyphenyl rings of EGCG within 4 Å from the solvent exposed Trp160 in hF, which is important for understanding the aforementioned changes in the fluorescence and CD spectra of OVA.

Taken together, our *in silico* analysis suggests that OVA contains at least one putative high-affinity EGCG-binding site, which is positioned near the major fluorophore in the allergen.

3.4. The EGCG binding to OVA does not prevent activation of basophils in the patients allergic to eggs

Changes in the protein conformation and surface hydrophobicity can mask IgE epitopes, which affect the interactions of allergens with antibodies and cell surface receptors. Consequently, changes in the protein structure could potentially modulate biological processes dependent on the surface recognition such as endocytosis in the antigen-presenting cells. To assess if the binary OVA/EGCG complex modulates biological processes, we performed the IgE-binding inhibition and basophil activation assays.

The IgE-inhibition assay was employed to determine whether EGCG inhibited specific interaction between OVA and IgE antibodies from the sera of egg-allergic patients. Our data clearly showed that EGCG did not prevent the IgE–OVA interaction (Fig. 3A) suggesting EGCG-binding site on OVA is distinct from that of the IgE-binding site and/or of lower affinity compared with OVA–IgE interactions. More importantly, the same results were obtained irrespective of the EGCG concentration used in the experiment (Fig. 3A). The same results (findings) were obtained using ELISA (data not shown).

In the basophil activation assay, the goal was to assess whether the OVA/EGCG complex retained the ability to cross-link the OVA-specific IgE bound to basophils from egg-allergic patients *ex vivo*. This interaction would trigger cross-linking of the Fc ϵ 1 receptor, basophil degranulation, and lead to expression of CD63 and CD203c on the cell surface. Our results show that OVA efficiently cross-links IgE bound to receptors on basophils specifically and induced anaphylactic degranulation of cells in two egg-allergic

patients tested in the presence of EGCG (Fig. 3B). In addition, the level of basophil activation associated with OVA alone did not differ significantly when compared with basophils treated with OVA and increasing concentrations of EGCG (Fig. 3B). EGCG alone did not affect activation of basophils in the egg-allergic patients (SOM Fig. S2A) nor did it augment the OVA-mediated basophil activation in non-responders (SOM Fig. 2B).

3.5. Uptake of OVA by monocytes is inhibited by EGCG

Monocytes and other immune cells that can serve as the antigen-presenting cells (APC) represent suitable models for assessing protein uptake and processing of modified allergens and allergens associated with bioactive compounds or polymers (Marengo et al., 2011). Modifications in the allergen structure could modulate the allergen uptake by APCs and influence the fate of the allergic immune response as already demonstrated for Bet v1 fold variants (Kitzmüller et al., 2012). To facilitate efficient capture of the molecule, antigen uptake by APCs, including monocytes, is mediated by several endocytic mechanisms (Thumann et al., 2003).

To establish if EGCG could inhibit OVA uptake, we performed the monocyte uptake experiments. The CD14⁺ monocytes were purified from PBMCs by positive selection and incubated with FITC-labelled OVA or OVA/EGCG in different ratios for up to 3 h. To ensure that increase measured in mean fluorescence intensity (MFI) was due to OVA internalisation, we used controls where cells were kept at +4 °C for the same period of time in the presence of FITC-OVA.

As shown in Fig. 4, freshly isolated monocytes took up FITC-OVA efficiently. In the presence of EGCG, there was a decrease in MFI of cells incubated with EGCG and FITC-OVA after 3 h compared with cells incubated with FITC-OVA alone. MFI of cells kept at cold (+4 °C) was the same as at $t = 0$ indicating increased fluorescence was due to FITC-OVA internalisation (Fig. 4A). When analysing the slopes of linearised uptake curves (Fig. 4B, Table 2), the differences obtained demonstrated slower uptake of OVA by monocytes in the presence of EGCG. This evidence, in addition to the ability of the OVA/EGCG complex to stimulate basophil degranulation, provides better insight into the mechanism underline how polyphenol–allergen interactions might modulate immune response.

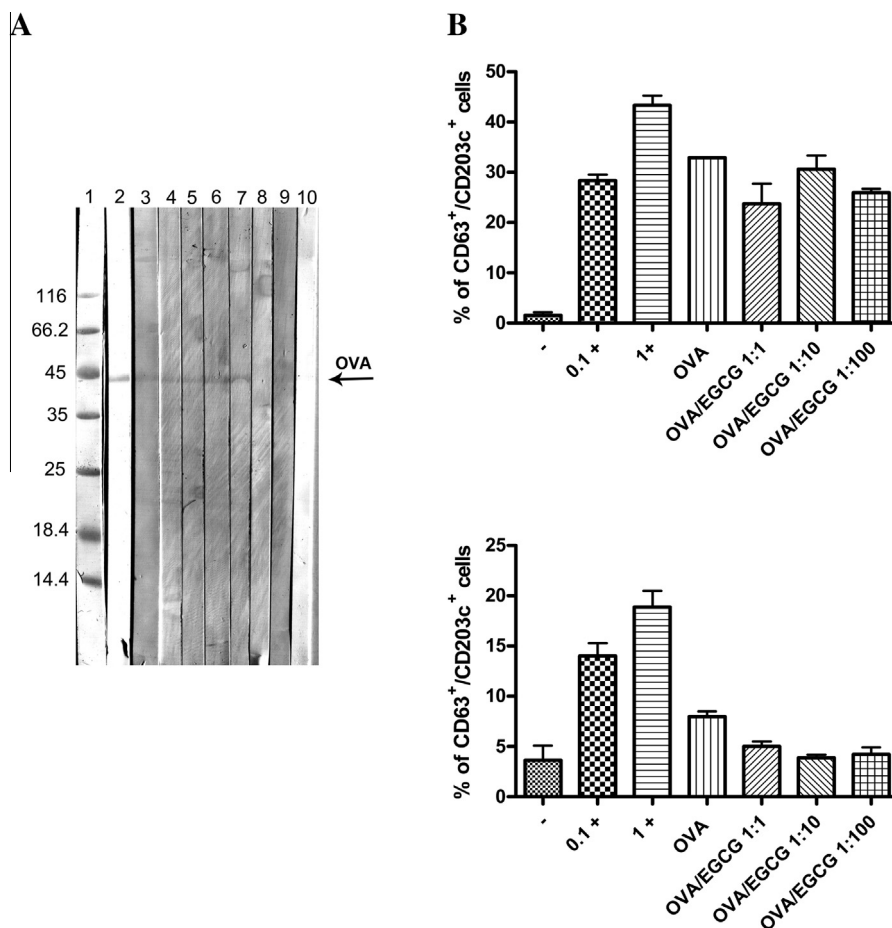


Fig. 3. The basophil activation by OVA in the presence of EGCG and the IgE-OVA inhibition assay. (A) EGCG does not inhibit interactions between pure OVA and IgE antibodies isolated from the serum of allergic patients in immunoblot. Lane 1: Molecular weight markers; Lane 2: stained with Ponceau S, Lane 3: OVA with positive serum; Lanes 4–7: varying EGCG concentrations (1, 5, 10, 15 and 100 μ M); Lane 8: 100 μ M EGCG and the positive serum; Lane 9: OVA/EGCG in the absence of serum; Lane 10: OVA/EGCG (100 μ M) and the negative serum. (B) Immediate effects of EGCG addition to food allergens did not prevent degranulation of basophils of allergic patients. With basophil activation test we have determined the percentage of basophils which express CD63 and CD203c as activation markers, by means of flow cytometry, after *in vitro* stimulation with OVA and OVA/EGCG complexes, using double labelling with monoclonal antibodies anti-CD63-FITC and anti-CD203c-PE. This experiment was performed using sera from two patients and results are shown in the corresponding graphs (top and bottom).

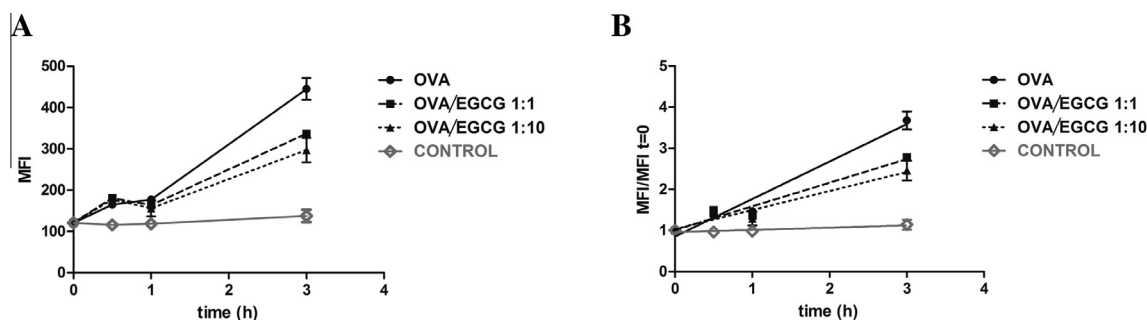


Fig. 4. EGCG decreases the rate of the FITC-OVA uptake by human monocytes isolated from non-allergic patients. (A) MFI (see definition in the text) of CD14⁺ monocytes was measured by flow cytometry after 0, 0.5, 1, 3 h of uptake. (B) Analysis of the uptake kinetics: linear regression of data was achieved by dividing MFI value of samples at $t = 0.5$, 1, 3 h with the average MFI value at $t = 0$ h. Data are obtained from one out of three blood donors. Ratios 1:1 and 1:10 represent final OVA:EGCG molar ratios.

4. Discussion

Interaction of EGCG with various proteins is now widely accepted and proposed to contribute to its diverse biological activities. This report provides insight into interactions between OVA and EGCG by fluorescence, CD spectrometry, ITC, and molecular docking. Biological consequences of the OVA/EGCG complex were determined by analysing the inhibition of IgE binding from the sera

of egg-allergic patients (Western blot), the capacity to elicit basophil activation (basophil activation assay), and monitoring allergen uptake by freshly isolated CD14⁺ monocytes. Our study revealed a new molecular mechanism for EGCG action, which affects both immunogenicity and the structure of OVA, in particular.

Both high-affinity binding and non-specific adsorption of polyphenols to proteins are of importance in complex food systems. Because low concentrations of EGCG are more likely to occur at

physiological conditions, the biological significance of the high-affinity non-covalent binding of EGCG to globular dietary proteins, in this case OVA, has been investigated using fluorophore quenching method (Xiao et al., 2011). In our study, the K_a determined using the fluorophore quenching method was in the range of 10^4 L mol⁻¹, which is similar to the binding affinity of EGCG to diary proteins (Hasni et al., 2011; Kanakis et al., 2011), and to that of the purine alkaloids (i.e. caffeine) to OVA (Wang et al., 2013). However, because of the apparently high molar ratio required for the full saturation in the ITC experiments (Fig. 1C), EGCG almost certainly “non-specifically” adsorbs to the surface of OVA in addition to binding to specific high-affinity sites. This conclusion would support the hypothesis suggesting the surface adsorption mechanism of polyphenols is responsible for protein aggregation and precipitation (Dobreva et al., 2011).

Our *in silico* calculations suggested the existence of a putative high-affinity site, which may be occupied at low EGCG concentrations. The predicted high-affinity EGCG-binding site is located near Trp160. This particular Trp side chain is solvent exposed, and is located on the most exposed and dynamic secondary structure element in serpins (hF), which is often used to monitor conformational and structural changes in serpins. The binding of EGCG near Trp160 could explain why the fluorescence quenching effects and changes in the CD spectra were seen once EGCG was added to the OVA solution. Finally, the predicted EGCG-binding pocket only partly overlaps with the IgE recognition epitope, which again agrees with our observations that the addition of EGCG could not inhibit directly the binding of IgE to OVA. It is worth noting that the physiologically active purine alkaloid, caffeine, whose binding to OVA was recently demonstrated using fluorophore quenching (Wang et al., 2013), preferentially docks in the same binding pocket as EGCG according to our *in silico* calculations (see: Fig. S3). The interesting feature of the hydrophilic cleft in OVA to bind physiologically active ligands has not been reported previously, and it could point to a novel role of OVA in binding, protecting, and delivering hydrophilic cargo from the complex food systems to physiological targets. It is also important to note that our docking calculations and ITC measurements suggested additional low-affinity binding sites that could be physiologically important at higher ligand concentrations. One of the pockets is particularly interesting since it is positioned near Asn298, which is the sole N-glycosylation site in OVA (ligand IX, Fig. S1) (Sheares & Robbins, 1986).

Changes in the CD spectra and secondary structure content of OVA suggest subtle structural adjustments in the allergen on EGCG binding. Our measurements suggest that ~20 amino acids undergo a conformational change from random coil into the β -strand. The structural analysis revealed putative interactions of EGCG with the high-affinity pocket could promote changes in three loop regions: (i) the segment N-terminal of hB, (ii) the loop linking hD and s2A, and (iii) the loop between hE and s1A. Attainment of an ordered structure in the hD-s2A loop is quite plausible since similar restructuring has already been observed in a viral serpin CrmA. There, the hD-S2A loop adopted a β -strand configuration and was termed s1'A (Simonovic, Gettins, & Volz, 2000). Thus, the EGCG binding might affect not only overall conformation of the protein but also, and perhaps more importantly, the structure of the conformational IgE-binding epitopes.

However, for most food allergens, including OVA, the linear epitopes are more important for IgE binding than the conformational epitopes (Honma et al., 1996; Johnsen & Elsayed, 1990; Kahlert, Petersen, Becker, & Schlaak, 1992; Stanic-Vucinic et al., 2012). Our computational predictions suggest that these epitopes would be relatively insensitive to the EGCG-induced conformational changes in OVA. Indeed, both *in vitro* and *ex vivo* IgE-binding experiments imply the EGCG-binding site on OVA is distinct from

the IgE-binding sites. This is in agreement with the previously published studies which showed the major IgE binding epitopes in OVA are located near the N- (region 1; residues 24–33 and 56–182) (Honma et al., 1996; Johnsen & Elsayed, 1990; Kahlert et al., 1992) and C-termini (region 2; residues 307–391) with the immunodominant site located near RCL (residues 364–372) (Kahlert et al., 1992). The predicted EGCG-binding site in OVA is distinct and only partially overlaps with region 1 of the IgE-binding epitope. This crevice is deep and quite inaccessible compared to the rest of the IgE epitope. Thus, it is perhaps not surprising that EGCG does not affect IgE–OVA interaction to any great extent.

Because the EGCG-binding pocket does not overlap with the immunodominant IgE epitope, we conclude the influence of EGCG on the interactions between OVA and IgE are less important in food allergy than EGCG interference with various cell signaling pathways, receptor expression, and immune system regulation. For instance, interaction of EGCG with lipid rafts might influence endocytosis, which is essential for regulating numerous important cellular processes such as signal transduction, immune surveillance, antigen uptake and presentation, cross-presentation, and the maintenance of the cellular homeostasis.

The antigen uptake by APCs is usually mediated by several endocytic mechanisms, which increase the efficiency of the foreign structure and molecule capture (Mellman, 1996). OVA is commonly used as a model system to study the mannose receptor-mediated endocytosis of soluble molecules by immature dendritic cells (DC), which highly express this receptor. Both endocytosis and pinocytosis are involved in OVA uptake by the immature DCs (Hilmenyuk et al., 2010). OVA is also efficiently taken up by freshly isolated monocytes (Zhang et al., 2007) that do not express the cell-surface mannose-specific receptors. These cells take up antigens via fluid phase endocytosis albeit less efficiently than DCs.

Monocytes play an important role in the initiation, development and outcome of the immune response. They can differentiate into very potent antigen presenting DCs under suitable conditions. Immature DCs, on the other hand, exhibit pronounced macropinocytosis, coincident with the highly efficient antigen processing. It has been shown that EGCG is capable of efficiently impairing the endocytic uptake of FITC-dextran in the immature DCs, but not in the LPS-treated mature DCs (Yoneyama et al., 2008).

Furthermore, EGCG might be involved in regulating the ruffling process, which leads to macropinocytosis. Although the role of protein kinase C in regulating macropinocytosis is unclear, it is well established that it mediates endocytosis in neutrophils (Moraru, Laky, Stanescu, Buzila, & Popescu, 1990). Also, the products of phosphatidylinositol-3-kinase and phospholipase A2 that modulate actin polymerisation are implicated in ruffling. Intriguingly, it has been shown that both protein kinase C and phospholipase A2 are subject to inhibition by EGCG (Pignatelli et al., 2006; Van Aller et al., 2011). EGCG also inhibits internalisation of caveolae in the endothelial cells presumably by interacting with the cellular lipid milieu, lipid rafts (Zheng et al., 2009). In addition, EGCG binds to the cell surface raft-associated 67 kDa laminin receptor with high affinity and mediates actin remodelling by suppressing phosphorylation of the myosin II regulatory light chain (Fujimura et al., 2006). These findings combined with our observations, strongly indicate that EGCG might directly impair several distinct endocytic processes and uptake by APCs, including monocytes.

In conclusion, we provide further evidence in support of the proposed mechanism in which EGCG interactions with the food antigen OVA contribute to its diverse biological activities. Impairment of the OVA/EGCG uptake process by APCs almost certainly influences antigen trafficking and presentation by APCs, and may modulate the course of the immune response. Finally, based on our evidence and that of others, we speculate major dietary proteins might serve as previously unrecognised carriers for the

biologically potent EGCG and other polyphenols to cellular targets and cell-bound receptors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2014.05.005>.

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Title: Interactions of epigallo-catechin 3-gallate and ovalbumin, the major allergen of egg white

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Supporting On-line Material

Experimental part:

1.1. Fluorescence quenching analysis

Fluorescence quenching is described by Stern-Volmer equation (Eq. 1):

$$F_0/F = 1 + K_{SV} \times [Q] \quad (\text{Eq. 1})$$

where F_0 and F are the fluorescence intensities before and after addition of a quencher; K_{SV} is the Stern-Volmer quenching constant and $[Q]$ is the concentration of the quencher. The obtained Stern-Volmer plots were linear in all conditions, thus allowing the calculation of the fluorescence-quenching rate constant (k_q) using Eq. 2 and the known value of 10 ns for Trp τ_0 (fluorescence lifetime of fluorophore without a quencher) in biopolymers (Lakowicz, Gryczynski, Gryczynski & Dattelbaum, 1999; Wang et al., 2013):

$$K_{SV} = k_q \times \tau_0 \quad (\text{Eq. 2}) \quad (\text{Lakowicz et al., 1999})$$

For the static quenching, the binding constant K and a number of binding places can be calculated according to a double-logarithmic equation:

$$\log[(F_0 - F)/F] = \log K_a + n \log [Q] \quad (\text{Eq. 3}) \quad (\text{Lakowicz et al., 1999})$$

The slope of the double logarithmic Stern-Volmer plot yields the number of binding sites and the intercept provides the binding constant (K_a). The fraction of fluorophore accessible to solvent (f_a) can be calculated according to modified Stern Volmer equation:

$$F_0 / (F_0 - F) = (1 + (1 / K_{SV} \times [Q])) \times 1 / f_a \quad (\text{Eq. 4}) \quad (\text{Lakowicz et al., 1999})$$

The average values were used for calculation of binding parameters. Statistical analysis was performed using descriptive statistics tool available in Excel.

1.1. Isothermal Titration Calorimetry (ITC)

All ITC measurements were taken on MicroCal iTC200 (GE Lifesciences, USA). In a typical experiment, OVA solution (30 μM) was placed in the 1.4 mL sample cell of the calorimeter and EGCG solution (370 μM) was loaded into the injection syringe. EGCG was titrated into the sample cell as a sequence of 20 injections of 10 μL aliquots. The

time delay (to allow equilibration) between successive injections was 1 min. The contents of the sample cell were stirred throughout the experiment at 100 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat (μJ) against injection number and were then integrated to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs} , kJ mol^{-1}) against molar ratio. Control experiments included the titration of 370 μM EGCG into buffer. Corrected data refer to experimental data after subtraction of the EGCG into buffer control data. ITC data were analysed using the MicroCal ITC data analysis program.

1.2. Docking analysis and structural modeling of the OVA/EGCG binary complex

1.2.1. Protein structure preparation

All water residues and ligand atoms were removed from the structure. The protonation state of each titratable amino-acid was estimated using finite difference Poisson-Boltzmann (FDPB) continuum electrostatics methodology as implemented in H++ program (Anandakrishnan, Aguilar & Onufriev, 2012). To remove possible steric clashes between amino-acids side chains, the OVA structure was optimized in CHARMM program (version c35b1) using the CHARMM22 protein force field (Bernard R. Brooks, 1983). Optimization of OVA structure was carried out in 2,000 optimization steps (1,000 steps with steepest-descent algorithm followed by 1,000 steps with Newton-Raphson algorithm).

1.2.2. Ligand structure preparation

The structure was optimized with quantum chemical MP2 method using standard 6-31G** basis set in Gaussian03 program package (Frisch et al., 2004).

1.2.3. Docking study

Optimized structures of protein and ligand were further subjected to AutoDockTools program for docking preparation. All protein residues were kept rigid and all ligand bonds were set to be rotational. A grid box, with the dimensions 24 x 24 x 24 Å was used to accommodate the ligand to move freely during docking run. A total of 420 docking runs were produced in order to cover whole surface of ovalbumin protein. The docking study was carried out with AutoDock Vina program (Trott & Olson, 2010).

2.8. Basophil activation assay

Patients were selected on the basis of positive skin prick testing to eggs, a documented clinical history of food allergy, and positive *in vitro* testing to egg white: IgE levels assessed to egg white with ImmunoCAP[®] 100 system using the ImmunoCAP[®] code f1 (Pharmacia Diagnostics, Uppsala, Sweden) above 0.35 kAU L⁻¹. Basophil activation assay was performed as described previously (Boumiza, Debard & Monneret, 2005) and

experimental details are shown in SOM. Briefly, heparinized blood samples (3 mL) were taken from three egg-allergic pediatric patients. Blood aliquots (100 μ L) were incubated with OVA and OVA:EGCG complexes (5 μ g mL⁻¹ OVA and EGCG in a molar ratio 1:1, 1:10, 1:100), anti-IgE antibody as a positive control (1 μ g mL⁻¹), or PBS as a negative control for 15 min (37°C). After incubation, cells were washed in PBS and then incubated with 10 μ L of PE-labelled CD203c monoclonal antibody 97A6 and FITC-labelled CD63 monoclonal antibody for 15 min at room temperature. Thereafter samples were subjected to erythrocyte lysis with 2 mL of FACSTM lysing solution (BD Biosciences). Cells were then washed, resuspended in PBS, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, USA). The basophil marker CD203c was used to set a basophil gate including only CD203c⁺ cells. The collected cells were further analyzed for granule release using an anti-CD63 antibody. The magnitude of basophil activation (BA) was calculated as the percentage of CD63⁺ events among the gated basophils (CD63⁺/CD203c⁺ cells). Statistical comparisons were done using Wilcoxon matched pairs test and the Statistica 9.0 program.

2.10.1. FITC labeling of protein

Ovalbumin was conjugated to FITC isomer I (Sigma-Aldrich, Taufkirchen, Germany) following manufacturer's instructions with some modifications. Briefly, FITC stock solution was prepared by dissolving 1 mg mL⁻¹ in DMSO. To 1 mL of OVA solution (2 mg mL⁻¹) in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.0 50 μ L of FITC stock solution were added very slowly in 10 μ L aliquots, every 10 min, while gently and continuously stirring. Labeling reaction was stopped with 50 mM NH₄Cl and unbound FITC was removed by gel filtration on PD-10 pre-packed columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). FITC conjugated OVA concentration was determined by BCA assay (P K Smith, 1985).

Results

Figure S1

Figure S1. A modified Stern-Volmer plot for the OVA/EGCG complex.

Table S1. The binding parameters describing the OVA/EGCG complex determined by the fluorescence quenching method

Protein	f_a	$K_{sv} (M^{-1})$	n	$K_a (M^{-1})$	$K_d (M)$
OVA (Mean $\pm$ SE)	0.70 ± 0.04	$(3.90 \pm 0.30) \times 10^4$	0.94 ± 0.02	$(1.20 \pm 0.30) \times 10^4$	$(8.30 \pm 0.14) \times 10^{-5}$

Table S2 – A summary of top 12 EGCG structures docked in OVA

Structure	Energy (kJ mol ⁻¹)	Amino acids interacting with docked EGCG
I*	37.6	Ser59; Arg97; Asp98; Asn101; Thr104; Ser116; Tyr138; Arg139; Trp160
II*	32.6	Ser59; Arg97; Asn101; Thr104; Ser113; Tyr138; Arg139
III	32.6	Asn47; Thr211; Glu212; Gln213; Ser258; Arg387; Ser390
IV*	32.2	Thr104; Asn107; Tyr110; Ser111; Ser113; Gln164; Asn166
V*	31.8	Ser59; Asn91; Arg97; Asp98; Asn101; Thr104; Lys105; Tyr138; Arg139
VI	31.8	Lys216; Gln219; Arg290; Lys292; Glu339; Asn341
VII*	31.8	Ser59; Arg97; Asn101; Thr104; Glu136; Tyr138; Arg139
VIII*	30.5	Asn91; Arg97; Asp98; Asn101; Thr104; Lys105; Tyr138; Arg139
IX	30.5	His45; Asn171; Gln174; Glu295; Lys296; Asn298; Thr300; Gln331
X*	30.5	Asn107; Asp108; Ser111; Ser113; Lys191
XI*	30.5	Thr104; Tyr110; Ser111; Ser113; Asn166; Lys191
XII	29.3	Lys199; Glu201; Asp202; Ser350; Asp356

*- Designates structures bound to the strongest predicted EGCG-binding site

Figure S2 – Molecular docking suggests that OVA contains multiple putative EGCG-binding sites. (A) The majority of structural hits bind into the site formed between hD, hE, s2A, and s1A. The strongest binder is ligand I; the predicted binding energies for each ligand are shown in Table S1. The view is similar as in Fig. 3A. (B) The second group of structures bind along the central β -sheet A and around the RCL (top of the OVA molecule). Ligand IX binds near the N-glycosylation site in OVA, Asn298 (blue). (C) Ligand III binds on a side of the β -sheet A. The view is rotated $\sim 180^\circ$ clockwise around the vertical axis relative to A. OVA is colored beige (both the surface and ribbon diagrams), and ligands are red sticks. The ribbon diagram on the right hand side of each of the panels serves to orient the reader.

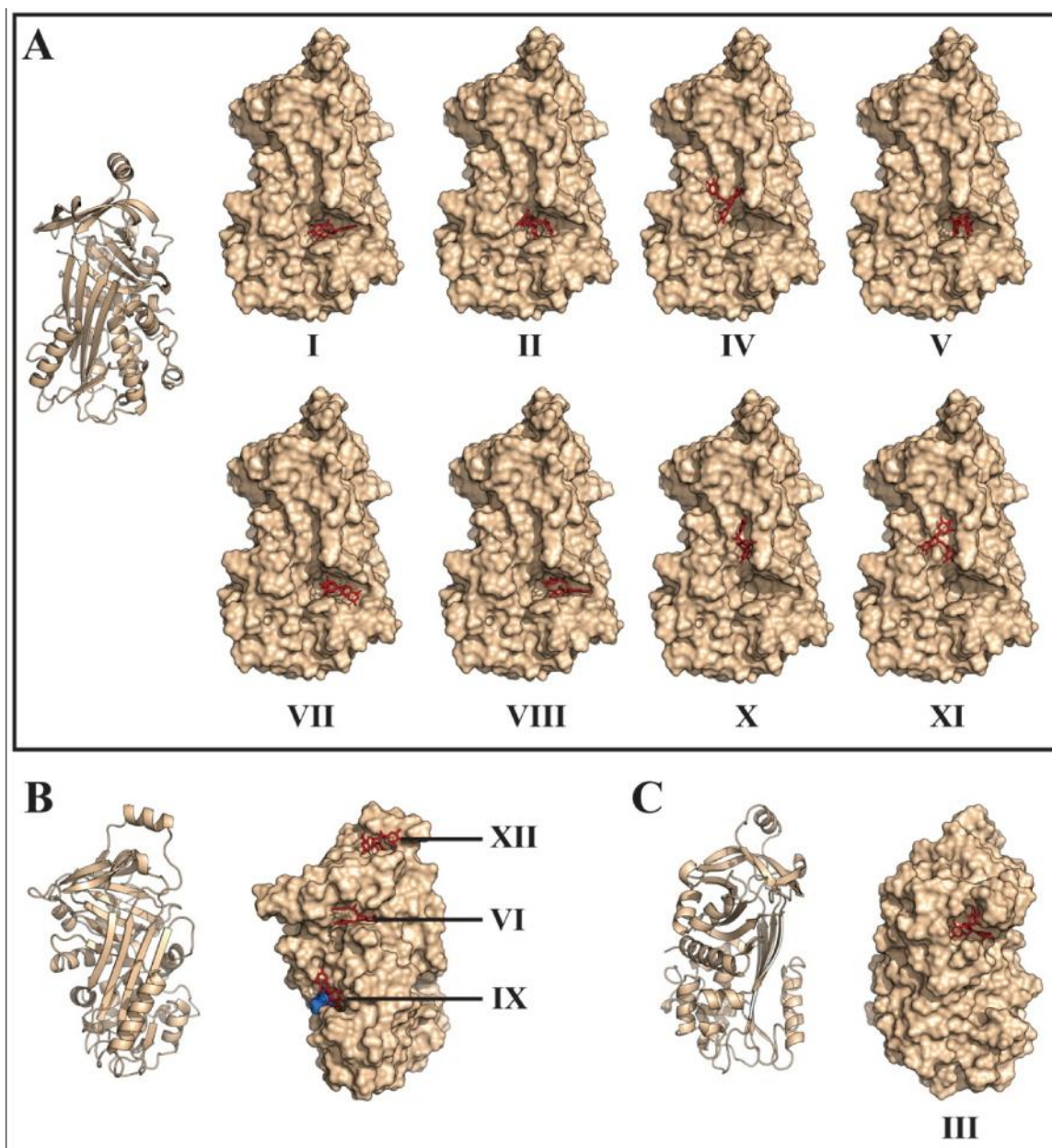
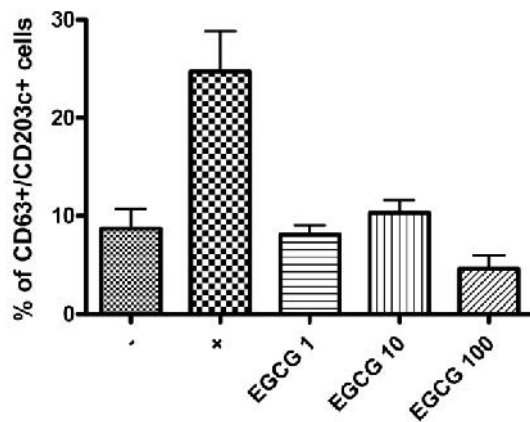


Figure S3. Basophil activation of allergic patients is not influenced by EGCG alone or augmented by EGCG in an egg allergic patient that does react to OVA. (A) EGCG alone does not stimulate degranulation of basophils from egg-allergic patients. Bars represent separate experiments: (-) media; (+) anti IgE antibody; (EGCG1, 10, 100) responses to increasing levels of EGCG that correspond to molar ratios shown under B and that are described in the main text. **(B)** Changes in % of activated basophils between OVA alone and OVA/EGCG complexes in egg-allergic patients were not detected regardless whether the patient reacted to OVA or not. Bars are the same as under A with the difference that EGCG was used in combination with OVA (ratios 1:1, 1:10 and 1:100 with the molar excess of EGCG). The level of basophile degranulation is plotted on the ordinate and shown as the percentage of the ratio of CD63 and CD203c positive cells in both panels. All measurements were done in duplicates.

A)



B)

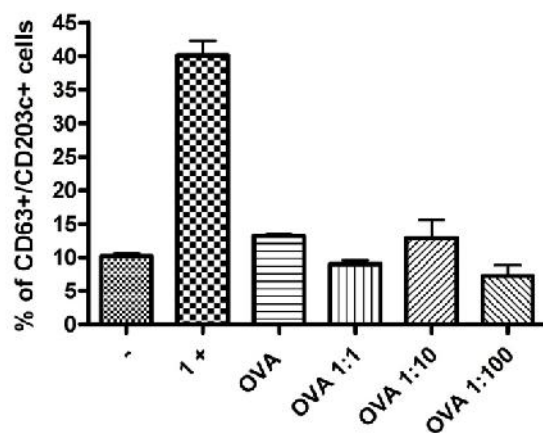
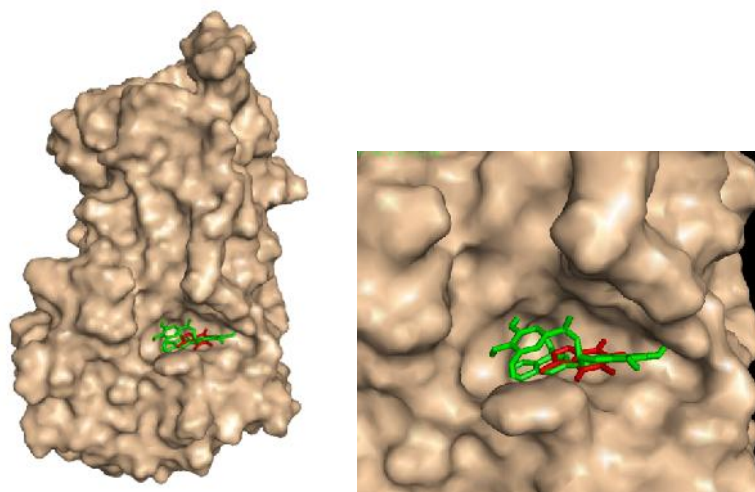


Figure S4. The predicted caffeine- and EGCG-binding pockets in OVA overlap.

(Left) A surface diagram of OVA (beige) with both caffeine (red sticks) and EGCG (green sticks) is shown. **(Right)** The close-up view of the predicted high-affinity pocket demonstrates that both compounds though structurally divergent preferentially dock to the same site of OVA. The caffeine structure was extracted from (Ishizu, Tsutsumi & Sato, 2009).



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