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Abstract

Ovalbumin (OVA), a major allergen from hen's egg albumen, tends to aggregate when heated. Depending on the balance of attractive and repulsive interactions, heat-induced OVA aggregates have various morphologies, which differ in digestibility. In the context of food allergy to egg, we investigated the ability of native and thermally aggregated OVA as well as their digests to induce the degranulation of a humanized rat basophil leukemia (RBL) cell line, which was sensitized with a pool of sera from egg-allergic children. Native and two thermally aggregated OVA forms were digested *in vitro* using a gastrointestinal digestion model based on the INFOGEST harmonized protocol including a final degradation with jejunal brush border membranes (BBM) enzymes. The course of digestion was monitored by the OPA method and by RP-HPLC. Digestibility was OVA small aggregates > OVA large aggregates >> native OVA and BBM peptidases only significantly hydrolyzed small-sized peptides from gastro-duodenal digests of the aggregates. The degranulation ability of the native OVA slightly changed during the gastric phase but mostly decreased during the duodenal digestion with no further change with BBM digestion. The degranulation ability of aggregates, which was significantly lower than the ability of native OVA, was not significantly affected by digestion. Digestibility and ability to induce basophil degranulation can thus not be straightforward linked.

Keywords

Allergenicity, digestibility, egg allergy, food structure, basophil degranulation

Abbreviations

A-L: large ovalbumin aggregates; A-s: small ovalbumin aggregates; EC50: protein concentration necessary to obtain 50% of the maximum mediator release; MaxD: maximum mediator release; N: native ovalbumin; OPA method: ortho-phthaldialdehyde method, OVA: ovalbumin; RBL: rat basophil leukemia; RP-HPLC: reverse phase - high performance liquid chromatography

1. Introduction

Food allergies affect between 2% and 10% of the worldwide population, with a steadily increasing prevalence over the recent years (Prescott et al., 2013; Sicherer & Sampson, 2010). Although resistance to digestion has been disputed for allergic risk assessment (Fu, Abbott, & Hatzos, 2002), food allergens must be at least partially resistant to digestion and be absorbed in an amount sufficient to activate the immune system (Bannon, 2004; Scheurer, Toda, & Vieths, 2015). Unquestionably, the digestion stability of large protein domains increases the possibility that massive amounts of foreign immunoactive polypeptides and the immune system encounter each other through the intestinal route. Regardless, the antigenicity of food antigens after digestion and intestinal absorption and how food processing affects these steps remain to be further understood (Moreno, 2007).

Hen's egg is one of the most common foods responsible for allergies in children, with a prevalence estimated at 1 to 2% in Europe (Xepapadaki et al., 2016). Eggs, present in a wide range of products, are generally consumed after heating, which could modify the structure of proteins, thus affecting digestion and allergenicity. Some children have been shown to react to both heated and raw eggs, whereas the majority tolerated extensively cooked eggs (Lemon-Mulé et al., 2008; Leonard, Caubet, Kim, Groetch, & Nowak-Węgrzyn, 2015). Ovalbumin (OVA), a major allergen of eggs, is, along with ovomucoid, the protein most widely studied when evaluating the link between thermal treatment and allergenicity. Heating OVA at 100 °C for 30 min (Martos, Lopez-Exposito, Bencharitiwong, Berin, & Nowak-Wegrzyn, 2011) increased its digestibility. Digestibility was also increased when OVA was heated at 90 °C for 15 min whereas treatment at a lower temperature (65 °C for 30 min) had no impact (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011). In parallel, it was shown that the digestion modified the antigenicity of the heated OVA by decreasing the basophil

activation ability (Martos et al., 2011) and the human IgE- or rabbit IgG-binding properties (Jiménez-Saiz et al., 2011; Joo & Kato, 2006). Depending on the chemical environment (e.g. pH, ionic strength, protein concentration) as well as on the kinetics of the heat transfer, OVA aggregates can have different morphological and structural properties. Thermal OVA aggregation and the structure of aggregates modulate the digestibility and the nature of peptides released at the end of an *in vitro* gastro-intestinal digestion (Nyemb, Guérin-Dubiard, et al., 2014). In a previous work carried out using a mouse model, we showed sensitization and elicitation abilities differed depending on the nature of the aggregates (Claude et al., 2017), but the antigenicity during digestion of such aggregated structures compared to native OVA has not been investigated.

Recently, Minekus et al. (2014) developed a static protocol of *in vitro* simulated digestion relying on physiologically relevant harmonized conditions. This protocol does not include a step simulating the small intestinal phase of degradation, which *in vivo* is carried out by brush border membrane (BBM) hydrolases at the level of the small luminal epithelium.

This study aimed to evaluate how thermal aggregation and aggregate morphology modulated the elicitation capacity of OVA during the digestion process. Native OVA and two types of OVA aggregates were compared when undigested and after sequential steps of simulated digestion (gastric, duodenal and jejunal) using a basophil activation assay with sera from egg-allergic children. A concomitant objective was to assess whether a jejunal phase with BBM enzymes needed to be included in digestion protocols when evaluating the degranulation ability of allergens.

2. Material and Methods

Unless otherwise stated, reagents from Sigma-Aldrich (Saint Quentin Fallavier, France) were used.

2.1. OVA samples

Aliquots of samples previously used to test the sensitizing capacity of native (N) ovalbumin and its large (A-L) and small (A-s) aggregates (Claude et al., 2017) were used. These aggregates had been prepared using the same OVA sample (OVA purified from EW kindly provided by INRA, UMR1253 “Science et Technologie du Lait et de l’Oeuf”, Rennes, France) and the same conditions (concentration, pH, ionic strength, temperature, time) during heating to be similar to those characterized by Nyemb, Guérin-Dubiard, et al. (2014). The mean particle sizes, determined by light scattering methods were about 50 nm for A-s, with a distribution size between 5 and 80 nm, and 65 µm for A-L, with a distribution size between 3 and 300 µm. Samples had been adjusted at pH 7.6, 0.15 M NaCl and 1 mg/mL and were stored at -20 °C until use.

2.2. Sera from egg-allergic patients

Sera from egg-allergic children with OVA-specific IgE were selected for this study (n=100). The sera belong to the registered sera collection (under the number DC-2008-809) implemented through clinical studies with the informed consent of patients or their caregivers and had been stored at -80 °C until use.

2.3. Measurement of IgE-binding to native and aggregated OVA in patients by F-ELISA

Allergen-specific IgE were determined using indirect fluorimetric-ELISA as previously described (Claude et al., 2016). All measurements were run in triplicate. Specific IgE bindings

were expressed by the ratios of mean fluorescence intensities measured with N, A-L or A-s (IF) on mean fluorescence intensity measured with PBS (IF_0).

2.4. *In vitro* digestion

The digestion protocol was based on the standardized model developed in the framework of the INFOGEST COST Action (Minekus et al., 2014) integrated with a simulated jejunal digestion using intestinal brush border membrane (BBM) enzymes. Two digestion protocols were performed, making use of short (30 min, 30 min and 2 hours) and long digestion times (2 hours, 2 hours and 5 hours) for gastric, duodenal and jejunal steps. The simulated gastro-duodenal digestion was stopped by adding 1mM of protease inhibitor (Pefabloc), or alternatively by heating in boiling water for 5 minutes, when samples were used downstream for BBM hydrolysis. BBM enzymes were prepared from pig jejunum and characterized according to Picariello et al. (2015). The aminopeptidase N (APN) and dipeptidyl IV (DPP-IV) activity was determined by spectrophotometric assays using L-leucine p-nitroanilide and Gly-Pro-4 nitroanilide as the substrates, respectively. The APN and DPP-IV activity was 879 µU/µL and 152 µU/µL, respectively. These values were roughly consistent with the value of 1018 µU/µL total peptidase activity, determined by reverse phase - high performance liquid chromatography (RP-HPLC) using angiotensin-I as the substrate (Picariello et al. 2015). Samples from gastro-duodenal digestion were incubated with BBM enzymes at ratio of 1:70 w/v, roughly consistent with the proteases-to-substrate ratio used in physiologically relevant simulated jejunal digestion (Shan et al., 2002). The reaction was stopped by heating samples in boiling water for 5 minutes. Digests taken at the end of each digestion step were frozen and stored at -20 °C until use.

2.6. Characterization of the digests

The extent of digestion was evaluated by measuring in triplicate the amount of free NH_2 in OVA samples using a modified ortho-phthaldialdehyde (OPA) method (Frister, Meisel, & Schlimme, 1988) with 2-dimethylaminoethanethiol hydrochloride as the thiol.

Gastro-duodenal and gastro-duodenal-BBM digests obtained at both short- and long-time digestion were separated by RP-HPLC using an HP1100 modular system (Agilent, Palo Alto, CA, USA) equipped with a C18 RP column of 2.0 mm id × 250 mm with a 4 µm particle diameter (Phenomenex, Torrance, CA, USA). Separation was carried out with a 5-60% gradient of solvent B over 60 min after 5 min of elution at 5% B. Solvent A was 0.1% (v/v) TFA in water; solvent B was 0.1% (v/v) TFA in acetonitrile. The column was equilibrated at 5% B. The column effluent was monitored by UV detection (220 nm). Before HPLC analysis, gastro-duodenal-BBM digests were purified from lipids included in the duodenal steps (phosphatidylcholine and bile acids). To this aim, 0.8 mL of -20° cold acetone was added to 0.2 mL aliquots of the digests and centrifuged (2500 x *g*, 4°C, 20 min) after vigorous shaking. The supernatant was discarded and the pellet was extracted again (twice) with 0.8 mL of cold acetone. The pellet was dried in speed-vac and finally dissolved in 0.2 mL of aqueous 0.1% (v/v) trifluoroacetic acid (TFA) and the peptide amount was determined using a kit for the modified micro-Lowry assay.

2.7. Activation of Rat basophil leukemia (RBL) cells by native and aggregated OVA in the presence of patient sera

G418-selected RBL-SX38 cells expressing human FcεRI, kindly provided by Pr Kinet (Harvard Medical School, New York, USA), were used as previously described (Claude et al., 2016) with a pool of sera, heated for 45 min at 56 °C in the presence of 4 M glucose to reduce

cytotoxicity (Binaghi & Demeulemester, 1983) and diluted 1:100. IgE-sensitized cells were stimulated in triplicate for 45 min at 37 °C (Blanc et al., 2009) by N, A-s or A-L diluted from 2 x 10^{-2} to 2 x 10^{4} ng/mL in Tyrode buffer containing 50% deuterium oxide. The release of β-hexosaminidase was measured as described previously (Bodinier et al., 2008). The spontaneous release was obtained with cells stimulated with the corresponding digestion control and total specific release after activation with human anti-IgE (Fc Region, Le27-NBS01, NBS-C Bioscience, Vienne, Austria). The percentage of degranulation was expressed by the ratio between the sample value and the specific value, both of which were corrected by the spontaneous release. The highest mediator release value (MaxD) and the allergen concentration corresponding to 50% of that highest release (EC50) were determined using GraphPad Prism 5.02 for Windows software (GraphPad Software Inc., La Jolla, CA, USA) by fitting a Hill 4-parameters model to the experimental data.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.02 for Windows software (GraphPad Software Inc., La Jolla, CA, USA), and *p* values below 0.05 were considered significant. Data are expressed as the mean ± SEM (standard error of mean). Data per group were first analyzed for normality using D'Agostingo and Pearson's omnibus normality test; next, differences between means/medians were analyzed by ANOVA/Kruskal-Wallis or 2-way ANOVA with subsequent Bonferonni/Dunn's post-tests (more than 2 groups).

3. Results

To compare the degranulation triggering ability of OVA digests, a relatively high volume of immunologically homogeneous serum was necessary. Thus, to constitute a pool of sera to be

used in the degranulation test with the digests, we characterized the pattern of IgE reactivity to N, A-s and A-L for 100 sera from egg-allergic children by ELISA.

3.1 IgE-binding to native and OVA aggregates in sera from egg allergic patients and selection for a pool.

When the data from this cohort were analyzed, the three OVAs exhibited significantly different IgE-binding capacities (Figure 1A): N had the highest and A-s the lowest, as illustrated by the mean ratios of fluorescence intensities IF/IF_0, which were set at 7.6 ± 0.4, 7.1 ± 0.5 and 5.1 ± 0.3 for N, A-L and A-s, respectively. However, IgE-reactivity was rather scattered, and four groups of sera could be identified as a function of IgE-binding to the OVAs. One third of the cohort, 33 sera, had similar IgE-binding to the 3 OVAs (variability lower than 25%). For roughly half of the cohort, namely, 54 sera, IgE-binding to N and A-L was similar and greater than to A-s. The remaining 13 sera showed higher IgE-binding to N than to A-L; for 7 among these latter, A-L and A-s exhibited similar reactivity, and for the remaining 6, the binding to A-L was greater than to A-s.

Sera were then selected to make a pool representative of the cohort, taking into account their availability, their ability to induce RBL degranulation with N and the observed variability of IgE-binding to N, A-L and A-s. The pool was thus constituted with 8 sera: 2, 4, 1 and 1 sera belonging to the 4 groups previously classified according to the IgE-binding behavior. Degranulation performed with the pool using N, A-L or A-s as an antigen is shown in Figure 1B. N, A-L or A-s induced the degranulation of the cells, though both aggregated forms resulted in a significantly reduced degranulation compared to the native form.

3.2 Characterization of the digests

Along the course of the simulated digestion, the content of NH_2 increased differently depending on the OVA (Figure 2). The overall increase was markedly lower for N than for the aggregates, and it was higher for A-s than for A-L both at short-times (Figure 2A) and long-times (Figure 2B) digestion. Whereas the extent of digestion was similar for the three OVA forms when the gastric phase lasted 30 min, both aggregates were significantly more hydrolyzed than N for the 1 h gastric digestion time. N was mainly digested during the duodenal phase, with no significant further increase induced by the BBM phase in both tested digestion times. Thus, the degree of hydrolysis in gastro-duodenal-BBM digests of N was not significantly affected by digestion times. The degree of hydrolysis of the aggregates significantly increased during both the duodenal and the final phase with the BBM enzymes, especially when this latter lasted 5 hours. In both times conditions, A-s were more extensively hydrolyzed than A-L following simulated duodenal or jejunal phases.

The RP-HPLC analysis of short-times gastro-duodenal digests demonstrated that the OVA aggregates contained a series of small sized peptides which were missing in the N counterpart (Figure 3A), in agreement with the OPA-based determination of -NH_2 groups. For example, the peaks at 21.4, 24.1 or 27.1 min, most likely due to small-sized peptides based on the retention time, were detected only for the aggregates, whereas less abundant peaks at 20.1 and 26.2 min were mainly found in the N-digests. The most intense peak eluting around 30 min, common to the three OVA forms, was the Pefabloc inhibitor used to stop the proteolysis of the duodenal step. Other peptides (e.g., at 45.3 and 50.1 min) were produced in digests from the three forms (Figure 3A). The detection of a low variety of different peptides after gastro-duodenal digestion is consistent with the relatively low degree of hydrolysis observed at this level. Notably, a broad RP-HPLC peak of undigested native OVA centered at 60.1 min (Figure 3A) was still detectable after gastro-duodenal

digestion (short incubation times), differently from the aggregates. An intense peptide peak specific to the N digest was detected at 33.9 min (along with smaller ones, such as at 18.3 and 46.1 min) in the gastro-duodenal-BBM digests (long incubation times, Figure 3B), whereas chromatograms of digests of the two aggregates were similar, with specific peaks at 10.6, 12.7, 22.4, 29.7 and 32.3 min, only differing by intensity. The large peak at 26.6 min and those at 10.6 and 20.8 were free tryptophan (W), phenylalanine (F) and tyrosine (Y), respectively, as confirmed by HPLC comparison with authentic standards; free aromatic amino acids were practically missing in the RP-HPLC chromatogram of N digests. In general, a low number of peptides survived the complete digestion of the aggregates, also including the degradation with BBM enzymes, which releases a large amounts of free amino acids not detected by HPLC (except for the aromatic ones).

Overall, these results indicate that N is only partially degraded at the gastro-duodenal level and due to the upstream formation of only large-sized polypeptides, the activity of jejunal BBM peptidases was very limited. For both aggregated OVAs, the gastro-duodenal degradation steps produced significant amounts of small-sized peptides, which were susceptible of further hydrolysis by BBM peptidases. The duodenal and jejunal hydrolyses of A-s was more pronounced than for A-L OVA.

3.3 Impact of the digestion on the ability of native and aggregated OVA to induce the RBL-SX38 cell activation

The RBL-SX38 activation assay was performed using N, A-L or A-s as the antigen at different steps of digestion with the pool of 8 sera from egg-allergic children. Limited volumes of sera from children allowed us to perform this study with digests obtained with short incubation times. The comparison at different steps of the *in vitro* simulated digestion for each form of

OVA clearly showed that digestion affected the degranulation ability of N (Figure 4A) more than abilities of the aggregates (Figure 4B and 4C).

Gastric digestion modified the degranulation curve of N with a significant decrease of the Dmax value (Fig. 4D), whereas the duodenal degradation largely decreased the degranulation percentage for a fixed OVA concentration and resulted in a significant increase of the EC50 (Figure 4E) without further changes after BBM hydrolysis. The degranulation abilities of both aggregates did not significantly change at any step of simulated digestions (Figures 4D and E).

These results demonstrated that the abilities to activate basophils of N and aggregated OVA were differently affected by the digestion process.

4. Discussion

To elicit an allergic reaction, an allergen must reach the immune cells in a form that is able to activate them. There is still limited information about the allergenicity of heated protein during digestion. For most food allergens, IgE-binding decreased with thermal processing (Verhoeckx et al., 2015). Undigested A-s displayed lower IgE-binding capacities than both N and A-L OVA. However, this was the mean behavior displayed by the sera from a large cohort of egg-allergic children and we also found that, probably linked to the IgE-repertoire of the sera, various patterns of IgE-binding to the OVAs could be detected. Resistance to digestion by itself is not sufficient to predict the allergenic potential (Bøgh & Madsen, 2016; Moreno, 2007). On the other hand, thermal aggregation primarily affects digestion susceptibility, with IgE-binding being generally decreased by digestion more effectively for heated than raw allergens, as for OVA heated at 90 °C for 15 min (Jiménez-Saiz et al., 2011). In agreement with previous work (Nyemb, Guérin-Dubiard, et al., 2014; Nyemb, Jardin, et al.,

2014), we found that A-s was more susceptible to digestion than A-L and both aggregates more than N. Beyond IgE-binding capacity, we aimed at evaluating how the digestion changed the degranulation capacity with a pool of human sera. When tested with the pool composed of 8 sera selected according to the observed variability in IgE-binding to native and aggregated OVA, both aggregates had similar lower degranulation ability than the native OVA. In every digestion step, N, A-L and A-s always maintained some ability to induce basophil degranulation. Martos et al. (2011) found that digests (1 hour pepsin and 2 hours duodenal digestion) of heated OVA (30 min in a boiling bath) had a lower ability to activate human basophils than did digests of native OVA. Interestingly, the degranulation ability of N, which was more digestion-stable, was more affected by short-time gastro-duodenal digestion than were aggregates. In turn, aggregates exhibited a degranulation ability substantially unmodified by digestion. These results confirm the absence of a straightforward link between digestibility and the ability to induce basophil degranulation. It is clear that the extensive disappearance of a protein during digestion does not coincide with the complete degradation of antigenic peptides. Our data suggested that N contained some structural domains responsible of its degranulation ability that were mainly resistant to the gastric digestion but sensitive to the duodenal enzymes, or that were modified during heating. Finally, BBM enzymes appeared to have no significant effect on the degranulation ability for the three OVA forms, most likely because BBM hydrolysis is limited to shorter-sized peptides that had already lost the antigenic properties at the level of duodenal degradation. In contrast, large-sized peptides (more than 20 residues), which are most responsible for the degranulation properties, appear only minimally or not at all affected by BBM peptidases (Picariello, Ferranti, & Addeo, 2016). Nevertheless, the peptide degradation clearly progressed during the BBM hydrolysis of both aggregates, as demonstrated by a

297 noticeable release of free aromatic amino acids (especially tryptophan and tyrosine), which
298 was not mirrored by native OVA. We could assume that BBM enzymes mostly affected short
299 peptides with no degranulation capacities. From a methodological point of view, these
300 results suggest no need to integrate the harmonized INFOGEST digestion protocol with an
301 intestinal phase based on BBM enzymes, for the purpose of determining the degranulation
302 capacity of native and aggregated OVA during the digestion. Clearly, this issue should be
303 evaluated for other allergens. Moreover, no generalization can be inferred related to the risk
304 assessment of protein allergenicity, because the jejunal digestion with BBM enzymes might
305 play a critical role during the sensitization phase. To this aim, in the wide perspective of the
306 immunogenic mechanisms of food allergies, it remains to definitely establish the nature of
307 the allergenic determinant(s) and the body site where allergens and immune system come in
308 touch. However, both the higher degranulation capacity of undigested native OVA and its
309 late reduction during the duodenal digestion step could explain the lower tolerance of native
310 than heated OVA by most of the egg-allergic patients.

311 Further investigations are needed to complete these preliminary results and provide
312 information about the effect of OVA aggregation on its degranulation ability during the
313 digestion. Identification of the OVA entities responsible for the activation of basophils and
314 comparison with those resistant to human digestion reported by Benedé et al. (2014) will
315 allow us to provide novel information about allergenicity. This identification would require
316 the development of a specific methodology to target peptides with more than 20 residues,
317 longer than those typically analyzed by a peptidomics approach, which would be applicable
318 in general also to different allergens.

To summarize, this study highlights some features between the aggregation of OVA and its degranulation capacity during the digestion process. We showed that all forms of OVA maintained some ability to induce cell degranulation after short-time digestion. In these conditions, digestion had no effect on the ability of the thermal aggregates, whereas the degranulation ability of native OVA was decreased by gastro-duodenal digestion. BBM digestion did not show any supplemental effect. Overall, the thermal aggregation of OVA enhanced its digestibility, with a greater effect for small than for large aggregates, even though the two aggregates did not exhibited different degranulation ability.

Conflict of interest statement

The results were presented in poster P83 at the 5th International Conference on Food digestion in Rennes, 4-6 April 2017.

The authors have declared no conflicts of interest.

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Captions for figures

Figure 1: IgE-binding and basophil degranulation ability of native ovalbumin (N, squares), large aggregates of ovalbumin (A-L, triangles) and small aggregates of ovalbumin (A-s, diamonds). (A) Human IgE binding to N, A-L and A-s was detected with 100 sera from egg-allergic children by indirect ELISA assay. The results are expressed as the ratio of fluorescence intensities measured to N, A-L or A-s (IF) on the fluorescence intensity measured to PBS (IF_0). Bars stand for mean ± SEM, and the results of a statistical comparison for paired data by Friedman test and Dunn's post-test are shown by *** $p < 0.001$ and ** $p < 0.01$. Data from the 8 sera used to prepare the pool are drawn in black. (B) Basophil activation assay with the pool of the 8 selected human sera. RBL-SX38 cells were stimulated with N, A-s or A-L at concentrations from 2×10^{-2} to 2×10^{4} ng/mL.

Figure 2: Extent of digestion at short (A) and long digestion times (B) of native ovalbumin and large and small aggregates of ovalbumin as evaluated by the OPA method. The results of 2-way ANOVA and Bonferroni post-tests are shown above the bars (bars with the same letters are not significantly different at $p = 0.05$, the interaction was significant at $p < 0.001$).

Figure 3: RP-HPLC analysis at 220 nm of OVA gastro-duodenal (GD) digests at short digestion times (A) and OVA gastro-duodenal-BBM (GD-BBM) digests at long digestion times (B). Native ovalbumin (blue line), large aggregates of ovalbumin (red line) and small aggregates of ovalbumin (green line). W: free tryptophan, Y: free tyrosine and F: free phenylalanine.

Figure 4: Comparison of the basophil activation abilities of native ovalbumin (A), large aggregates of ovalbumin (B) and small aggregates of ovalbumin (C) before digestion (ND, dark solid line) and after gastric (dark dotted line), duodenal (light solid line) and BBM (light dotted line) digestion steps. Variations of Dmax (D) and LogEC50 (E) and results of 2-way ANOVAs and Bonferroni post-tests; only significant differences are drawn (interactions were significant at $p < 0.001$, letters show significant differences between OVAs at one digestion step, and stars show significant differences between consecutive digestion steps for each sample, ***: $p < 0.001$).

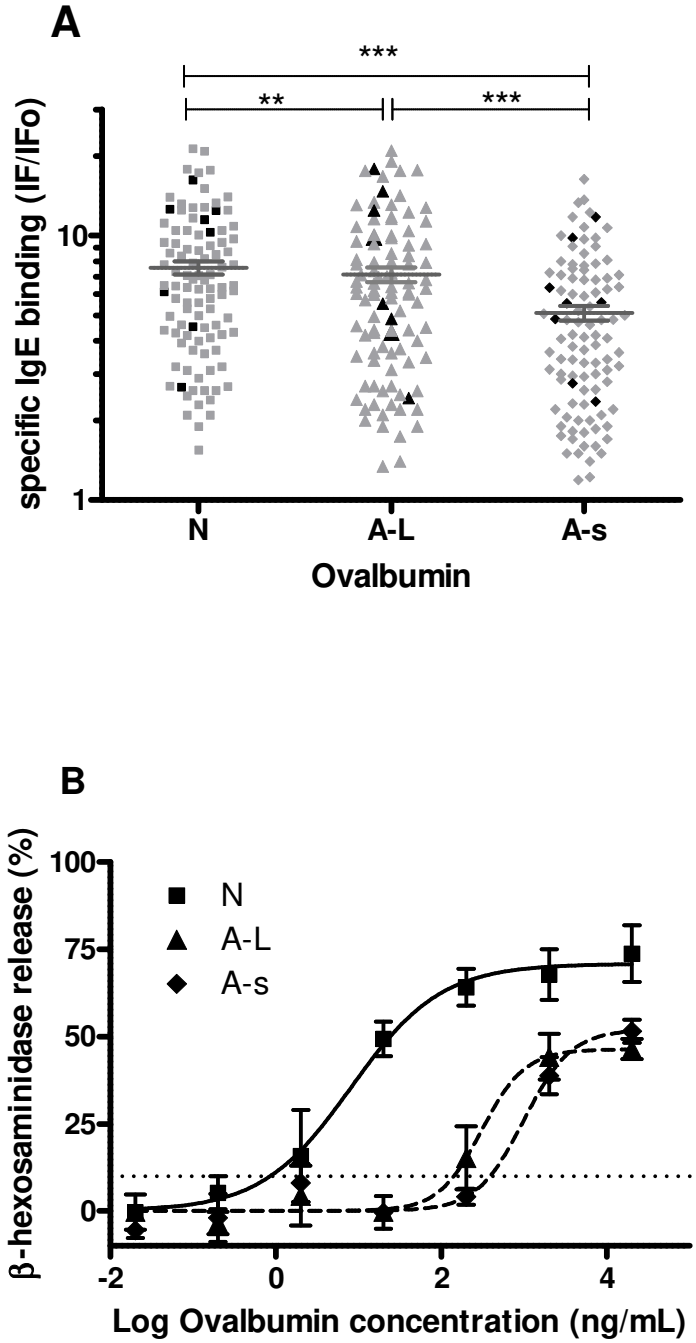

A
***
**
***
specific IgE binding (IF/IFo)
10
1
N
A-L
A-s
Ovalbumin
B
β-hexosaminidase release (%)
100
75
50
25
0
N
A-L
A-s
-2
0
2
4
Log Ovalbumin concentration (ng/mL)

Figure 1

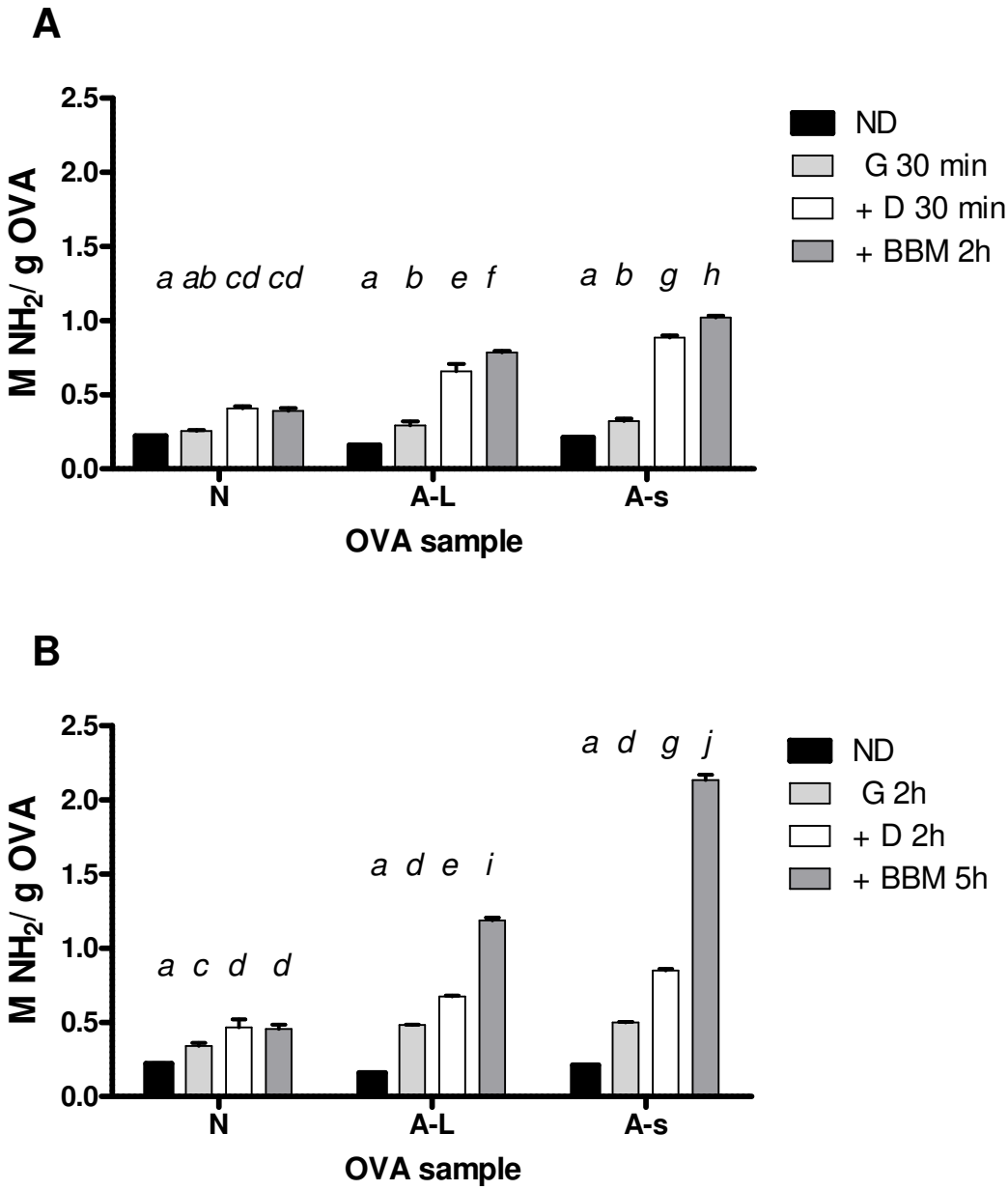
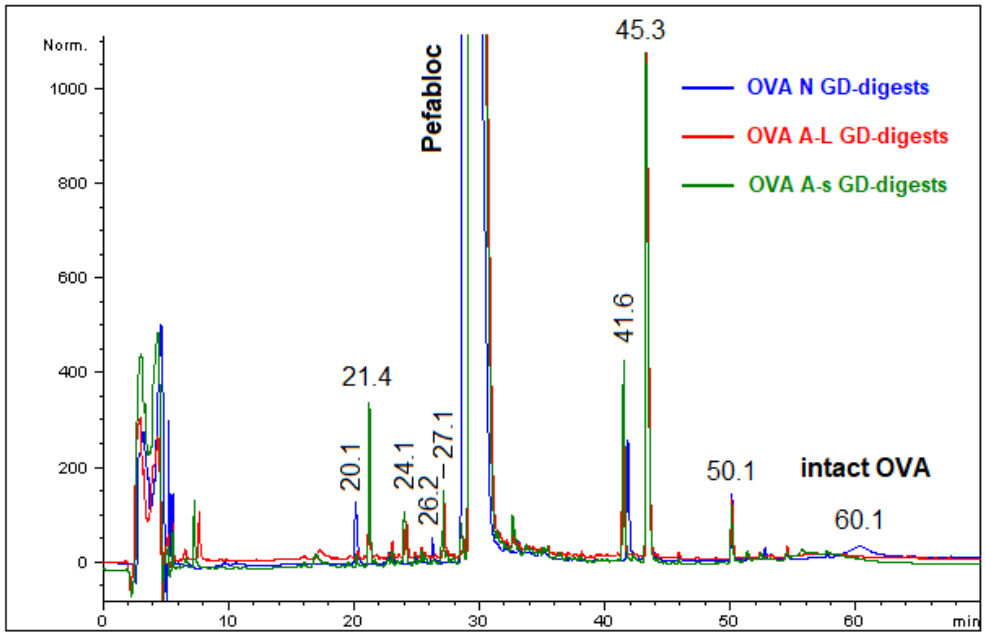

A
2.5
2.0
1.5
1.0
0.5
0.0
M NH2/ g OVA
a ab cd cd
a b e f
a b g h
N
A-L
A-s
OVA sample
ND
G 30 min
+ D 30 min
+ BBM 2h
B
2.5
2.0
1.5
1.0
0.5
0.0
M NH2/ g OVA
a c d d
a d e i
a d g j
N
A-L
A-s
OVA sample
ND
G 2h
+ D 2h
+ BBM 5h

Figure 2

A


Norm.
1000
800
600
400
200
0
Pefabloc
45.3
41.6
21.4
20.1
24.1
26.2
27.1
50.1
intact OVA
60.1
OVA N GD-digests
OVA A-L GD-digests
OVA A-s GD-digests
0
10
20
30
40
50
60
min

B

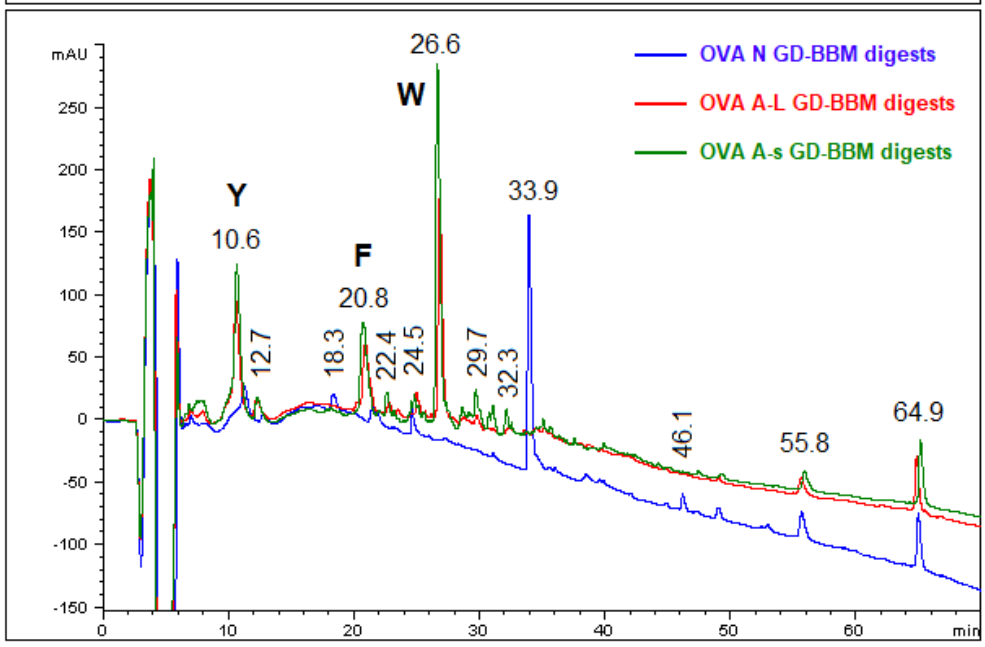

mAU
250
200
150
100
50
0
-50
-100
-150
26.6
W
Y
10.6
F
20.8
33.9
12.7
18.3
22.4
24.5
29.7
32.3
46.1
55.8
64.9
OVA N GD-BBM digests
OVA A-L GD-BBM digests
OVA A-s GD-BBM digests
0
10
20
30
40
50
60
min

Figure 3

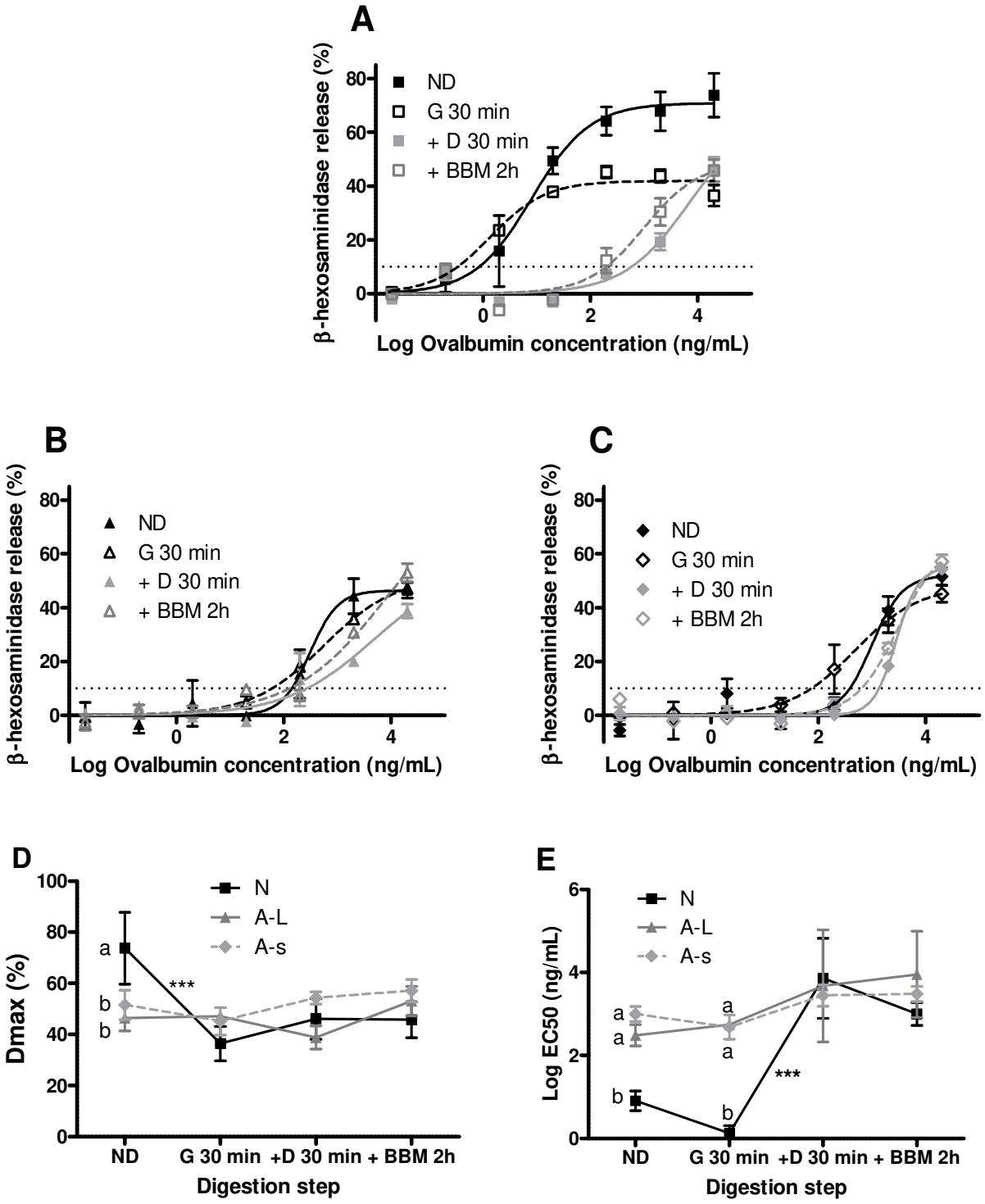

A
β-hexosaminidase release (%)
ND
G 30 min
+ D 30 min
+ BBM 2h
Log Ovalbumin concentration (ng/mL)
B
C
D
Dmax (%)
N
A-L
A-s
***
Digestion step
ND
G 30 min
+D 30 min
+ BBM 2h
E
Log EC50 (ng/mL)

Figure 4