Thermal treatment reduces gliadin recognition by ige, but a subsequent digestion and epithelial crossing permits recovery

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Abbreviations

MaxD: maximum mediator release; FAW: food allergy to wheat; RBL: rat basophil leukemia; Pro: proline; Gln: glutamine; LLS: laser light scattering; RP-HPLC: reverse-phase high-performance liquid chromatography; TFA: trifluoroacetic acid; ACN: acetonitrile; PVDF: polyvinylidene difluoride; HRP: *horseradish peroxidase;* DMEM: Dulbecco's modified Eagle's medium; TER: transepithelial electrical resistance; AS-FITC: Fluorescein-5-(and-6)-

sulfonic acid-trisodium salt; ELISA: enzyme-linked immunosorbent *assay*; PBS: *Phosphate-buffered* saline; GN:total gliadins; Gα: α-gliadins; HG0:heated gliadins; HhG5: heated and hydrolyzed 5 minutes gliadins; HhG15: heated and hydrolyzed 15 minutes gliadins ; HhG30: heated and hydrolyzed 30 minutes gliadins; HhG60: heated and hydrolyzed 60 minutes gliadins; HGα0: heated α-gliadins; HhGα5: heated and hydrolyzed 5 minutes α-gliadins HhGα15: heated and hydrolyzed 15 minutes α-gliadins; HhGα30 : heated and hydrolyzed 30 minutes α-gliadins.

Keywords: Gliadins, food allergy, aggregation, pepsin hydrolysis, Caco-2 transport, basophils degranulation.

Abstract

Wheat is one of the most important crops in the world in terms of human nutrition. With regards to health, some individuals exhibit wheat-related disorders such as food allergy to wheat (FAW). In this disorder, gluten is involved, particularly the gliadins which are among the main proteins responsible for FAW. Food processing, as well as digestibility and intestinal transport are key factors to consider since they may affect the allergenic potential of food allergens.

Wheat is always consumed after heat processing and this step may impact epitope accessibility by inducing aggregation and may irreversibly destroy conformational epitopes. Our aim was to investigate the effects of heating and digestion on the structure of well-known allergens (total gliadins and α-gliadins) and their capacity to maintain their allergenic potential after crossing an intestinal barrier.

The sizes of the processed (heated and heated/digested) proteins were characterized by laser light scattering and chromatographic reverse phase. The IgE-binding capacities of native and processed proteins were checked using a dot blot with sera from wheat allergenic patients. Furthermore, the abilities of these samples to cross the intestinal barrier and to induce mast cell degranulation were investigated by combining two *in vitro* cellular models, Caco-2 and RBL-SX38.

The heat treatment of total gliadins and α-gliadins induced the production of large aggregates that were hardly recognized by IgE of patients in dot-blot. However, after limited pepsin hydrolysis, the epitopes were unmasked, and they were able to bind IgE again. Native proteins (gliadins and α-type) and processed forms were able to cross the Caco-2 cells in small amount. Permeability studies revealed the capacity of α-gliadins to increase paracellular permeability. In the RBL assay, the total native gliadins were able to trigger cell degranulation, but none of their processed forms..However after crossing the CaCo-2 monolayer, processed gliadins recovered their degranulation capacity to a certain extent. Total native gliadins remained the best allergenic form compared to α-type.

1. **Introduction**

Millions of people consume products derived from wheat daily. Wheat is a source of carbohydrates (starch) and proteins, which constitute 65-75% and 8-15% of the dry weight of grain wheat, respectively (Rosell et al., 2014). Both are important for human' nutrition and livestock (Tatham & Shewry, 2012). Based on solubility, wheat proteins can be classified as water soluble albumins, salt soluble globulins, and prolamins insoluble in previously used buffers (Osborne T.B., 1924). This latter fraction is composed of gliadins and glutenins in approximately equal proportions (Thewissen, Celus, Brijs, & Delcour, 2011).

Both gliadins and glutenins make up gluten, whose viscoelastic properties are essential in determining dough processing properties, in particular, for the production of bread, pasta, and noodles (Shewry, 2009).

Gliadins represent up to 40% of wheat proteins. They are mostly monomeric proteins with molecular weights ranging from 28–55 kDa and can be divided, based on electrophoretic mobility, into α/β-, γ-, and ω-gliadins (Wieser, 2007; Shewry & Tatham, 1990). Alpha gliadins are the most abundant, accounting for 15-30% in most wheat cultivars (Li, Xin, Zhang, & Li, 2014). The gliadin structure consists of a central domain (CD) containing repetitive amino acid (AA) sequences rich in proline (Pro) and glutamine (Gln), and C-terminal non-repetitive domains which are more hydrophobic (Gianibelli et al., 2001; Shewry & Tatham, 1990).

Some individuals exhibit wheat-related disorders as celiac disease (CD) or allergies. CD is known to affect approximately 1% of Europeans, but reliable data on wheat allergy prevalence are lacking (Nwaru et al., 2014). However, the number of patients may be estimated to be 0.2-0.9% in adults and 0.4-1.3% in children (Czaja-Bulsa & Bulsa, 2017). Gliadins are among the main proteins responsible for both CD and wheat allergies (Ferretti et al., 2012). The allergic immune response is composed of two phases: the sensitization phase

with specific IgE production and binding to basophils and mast-cells, followed by the triggering and symptomatic phase with inflammatory mediator (histamine, β-hexosaminidase) release. Allergic symptoms may occur as immediate reactions within minutes to up to 2 h after ingestion of the offending substance (Thomas et al., 2006) and trigger various clinical manifestations such as anaphylaxis, asthma, urticaria, and digestive symptoms (Sicherer, 2000). The allergic reaction occurs through the interaction between antigens and immune cells, after food digestion and transport across gastro-intestinal track *via* paracellular or transcellular mechanisms. Some wheat proteins, including gliadins, display resistance to hydrolysis from gastrointestinal and brush-border membrane (BBM) enzymes (Shan et al., 2002).

Additionally, wheat-based foods are always consumed after cooking, which includes a heating step. Thermal treatments alter protein structures by unfolding them, with subsequent rearrangements of disulphide bonds occurring around 80-90°C and formation of aggregates above 90-100°C. In addition, their inclusion in a food matrix can induce chemical reactions such as Maillard reaction (Davis & Williams, 1998; Nicolai & Durand, 2013). Such modifications may affect epitopes by masking or destroying some of them or by inducing the formation of neo-epitopes, and thus may modify the allergenicity of proteins (Claude et al., 2016; Ilchmann, et al., 2010; Nakamura et al., 2006). This impact of thermal processing has been reported for hazelnut allergenicity, where roasting reduced the allergenicity, while the opposite situation was observed in peanuts, where the allergenicity was increased after roasting (Maleki et al., 2000).

In the case of wheat, Pasini et al. reported that bread baking resulted in wheat protein aggregation, and thus in the reduction of their digestibility (Pasini et al., 2001), as confirmed by Smith et al. (Smith et al., 2015). However, the IgE-binding capacity of bread was

comparable to that of dough, indicating the presence of remaining epitopes or the formation of neo-epitopes (Simonato et al., 2001).

Simonato and Pasini studies performed on the final products provided very important information on protein antigenicity, but they did not consider the destiny of food in the gastrointestinal track or its capacity to reach and activate the immune cells.

Considering the lack of literature concerning the transport of heated and heated/digested wheat allergens through intestinal epithelium, we chose to combine the processing of proteins with the capacity of samples to cross the intestinal barrier and trigger an allergic reaction. The intestinal barrier was modeled by monolayers of a human colon carcinoma cell line (Caco-2), and the capacity to trigger the reaction was estimated using the rat-basophil leukemia humanized model (RBL-SX38).

In this paper, we considered several events that food allergens undergo: heating, kinetics of hydrolysis under conditions close to those of the stomach, passage through epithelial cells and triggering capacity. Two wheat allergenic fractions, total gliadins and α-gliadins, were followed at the different steps. The native and modified proteins were characterized by laser light scattering (LLS) and by reverse-phase high-performance liquid chromatography (RP-HPLC). The effects of thermal treatment and pepsinolysis of the gliadin fractions on their IgE-binding or triggering capacity were investigated with sera from patients with FAW.

2. MATERIAL AND METHOD

2.1. Gliadins preparation

Crude total gliadins (GN) and α-type (Gα) made from the total gliadins fraction were purified by RP-HPLC from bread wheat flour cv Récital as described by (Popineau & Pineau, 1985).

2.2. Samples preparation: heat treatment and pepsin hydrolysis

Eighty mg of each sample was suspended in 4 ml of water and heated at 200°C for 20 min. Subsequently, 4 ml of 0.2 M acid acetic was added. The resulting pH was ≅ 3. For pepsin hydrolysis we chose a simplified model of digestion. Samples were placed at 37°C in a water bath with shaking, and pepsin (Sigma-Aldrich Saint Quentin Fallavier, France, P-6887, 178U/mg of proteins) was added (1:20E/S), this correspond to 3500 U ml^{-1} of porcine pepsine, which is in the range 2000 to 4000 U ml^{-1} as suggested by Minekus et al 2014 (Minekus, et al., 2014). The reaction was stopped at different times (0, 5, 15, 30 and 60 min for gliadins and 0, 5, 15, 30 for α-gliadins) by heating for 1 min at 100°C. Control (undigested, time=0) samples were treated in the same manner, but without pepsin addition. These samples were called HG0-HhG60 and HGα0-HhGα30 for heated (H) and hydrolyzed (h) gliadins and α-gliadins respectively. The samples were produced then lyophilized. The native proteins, gliadins (GN) and α-gliadins (Gα) were used as reference.

2.3. Human sera

Table 1 –Concentration of specific IgE against gliadins and α-gliadins, total IgE, measured by ELISA for each pool. Pool I, II, III were used in DotBlot experiments and pool IV, V were used in RBL-SX38 in vitro test. Pool VI was used as control.

IgE concentration (ng/ml)			
	Specific to glia	**Specific to α-glia**	**Total**
Pool I	106	111	1300
Pool II	43	72	6400
Pool III	27	27	nd
Pool IV	112	96	1500
Pool V	167	125	7500
Pool VI	0	0	1000

Sera were obtained from the Biological Resource Center (BB-0033-00038) of Clinical Immunology and Allergy Service of Angers University Hospital (France) with the informed consent of the patients. Control sera were obtained from healthy volunteers. Every pool was composed of three sera with comparable reactivity. The three groups used for dot blot analysis were characterized by a decreasing concentration in specific IgE from group I to III, with the group II exhibiting more IgE against Gα than against GN. The two groups used for

RBL-SX38 analysis were characterized by slightly higher amount of IgE against GN than Gα (Table 1).

2.4. Particle size analysis

The size distribution of HG0, HhG15 and HhG30 was determined by LLS using a Malvern Master Sizer M3002 (Malvern instruments, Malvern UK). Fraunhofer approximation was applied. Freeze dried samples were suspended in 0.1M acetic acid (Merck, Darmstadt, Germany) and analysed in triplicate with obscuration rate fixed at 8%. Particle size measurement range was between 0.02 -2000 µm. The mean particle size, corresponding to three repetitions, was calculated from the volume size distribution.

2.5. RP-HPLC

Analysis was carried out on an Alliance HPLC System (Waters, Saint-Quentin-en-Yvelines, France) using a C18 Nucleosil column (4.0 mm x 250 mm, 5 µm particle size, 300 Å pore size, Machery-Nagel EURL, France). Samples were solubilized at 1 mg/ml in eluent A (0.1% trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) / 5% acetonitrile (ACN) (Carlo Erba reagents, Val de Reuil, France) and filtered with a 0.45 µm PVDF filter prior to loading (50 µl). The elution was performed at a flow rate of 1 ml/min using eluent A and eluent B (0.08% (v/v) TFA in 85% (v/v). The separation was performed at 50°C using a 60 min gradient of 10-80% solvent B, and detection was carried out at 280 and 214 nm with a UV detector (Waters 2487, Saint-Quentin-en-Yvelines, France). Data were acquired and processed with Empower Software (Waters).

All experiments were performed in triplicate.

2.6. Detection of IgE-binding capacity through Dot Blot

Increasing amounts of each sample were spotted onto nitrocellulose a membrane (0.2 mm, Sartorius, Germany): 2, 4, 10, 20 µg for GN and Gα and 10, 20, 40, 60 µg for H and h

samples. The membrane was dried for 1 h at 37°C before incubation with IgE, according to the procedure described in Lupi et al. (Lupi et al., 2013). Pooled sera were prepared at a 1:20 dilution in washing buffer. The membrane incubation with polyclonal antibodies against the repetitive domain, N-ter and C-ter was performed as previously described (Lupi et al., 2013). The chemiluminescent substrate used for revelation was the Western Bright^{TM} Quantum chemiluminescence HRP substrate (ADVANSTA-K12042-D20, Menlo Park, USA), and a Fuji Las3000 (Fujifilm, France) camera was used for detection.

2.7. Transport of gliadins and α-gliadins across Caco-2 monolayer

Human intestinal Caco-2 (ATCC-HTB-37, Manassas, USA) cells were grown and maintained at 37°C, 95% humidity and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 mg/L) (BE12-733F, BioWhittaker Lonza, Levallois, France) as previously described by Bodinier et al. (Bodinier et al., 2007). Caco-2 cells were used at passage range from 30 to 50. Cells were seeded at $2.5x10^5$cells/ml onto 4.67 cm^2 polycarbonate inserts (3 µm pore diameter, Corning Costar) and were left to differentiate for 24 days; medium was replaced three times per week. Cell viability was assessed in previously experiment by using MMT assay as described in Bodinier et al. (Bodinier et al., 2007). The viability was checked for GN, HG0 and HG30 incubated for 24h in presence of 1mg/ml of proteins (data not shown). Epithelial integrity and maturity of the monolayers were checked by transepithelial electrical resistance (TER) monitoring using a millicell-ERS volt-ohm meter (Millipore) with "chopstick" electrodes (Millipore). TER values were expressed as Ohms (Ω)×cm2, taking into account the filter surface area. Only wells with TER values above 500 Ωxcm2 were used for protein transport tests.

On day 24, the DMEM was replaced with 1.5 ml and 2.6 ml of Ringer buffer (RB), pH 8 in the apical and basal compartment, respectively. The AS-FITC (Thermo Fisher Scientific, France) (100 µg/ml) in RB was added to the apical compartment and incubated at 37°C, 95%

humidity and 5% CO_2 for 1 h. Twenty-five µl was removed from the basal media every 20 minutes and used for determining the concentration of AS-FITC crossing to basal media. After this time, the apical compartments media were replaced by AS-FITC (100 µg/ml) and GN, HG0, HhG15, HhG30, Gα, HGα0, HhGα15, HhGα30 (1 mg/ml in RB) and incubated for 6 h under the same conditions. Controls were performed by adding AS-FITC (100 µg/ml) in RB without any protein, noted as C. Twenty-five µl were removed from the apical and basal media each hour and were used for determining the concentration of AS-FITC crossing to basal media over 6 h. The fluorescence was read at 485 nm. The concentration of AS-FITC was calculated using a standard curve of AS-FITC from 0 to 10 ng/mL. After 6 h, the remaining apical and basal media were collected to be used in the RBL-SX38 *in vitro* test described below. All experiments were done in triplicate. The results represented mean ± standard deviation.

2.8. quantification in apical and basal compartments by ELISA Inhibition

The amount of proteins moving from apical to basolateral media was estimated by an ELISA inhibition test. First, 96-well microplates (Nunc MaxiSorp, Fischer Scientific, Illkirch, France, Ref 442404) were coated with 5 µg/well of GN or Gα in 50 mM carbonate buffer pH 9.6 at a volume of 100 µl/well overnight, at room temperature (RT). All subsequent incubations were performed at 37°C. The coating solution was removed and 250 µl/well of blocking buffer and 2% (w/v) milk in PBS was added and incubated for 3 h. Microplates were washed with washing buffer, 0.05% (v/v) Tween 20, in PBS, after each incubation.

The competition was performed by adding apical (1:500) or basal (1:10) media to the anti-R gliadins domain antibody, also diluted in PBS-milk 0.1%. at 1:1000 v/v. After a 2 h incubation, these mixtures (100 µl) were added for 1 h to plates coated with GN or Gα. For the establishment of calibration curves, the competition was performed with a serial dilution of GN (0-10 µg/ml) or Gα (0-80 µg/ml), and the anti-R gliadins antibody at 1:1000 v/v.

Goat anti-rabbit IgG antibody (H+L) horseradish peroxidase conjugate human IgG adsorbed (BioRad, Marnes-la-Coquette, France, Ref 170-7515), diluted to 1:3000 in PBS, was incubated for 1 h. The colorimetric detection was performed as described in Lupi et al. (Lupi et al., 2013).

We quantified proteins only in the linear zone of the curve. Experiments were run in triplicate and repeated twice.

The percentage of inhibition was calculated as follows:

Inhibition: [(OD uninhibited – OD inhibited)/(OD uninhibited – OD buffer)]*100

Results were expressed as the percentage of protein measured in basal medium to the protein measured in apical medium.

2.9. **RBL-SX38 cell degranulation test**

The apical and basolateral mediums corresponding to the experiments performed with total gliadins (GN, HG0, HhG15, HhG30) and α-gliadins, (Gα, HGα0, HhGα15 and HhGα-30) were used to test the degranulation capacity using an RBL-SX38 *in vitro* model through the β-*hexosaminidase* release measure, as described by Blanc et al. (Blanc et al., 2009). RBL-SX38 cells expressing human FceRI, were kindly provided by Pr Kinet (Harvard Medical School, New York, USA). Antigen concentrations varied from 0.5 to 1000 ng/ml for α-gliadins samples and from 0.02 to 2000 ng/ml for total gliadins. Two pools of patient sera diluted 1:50 (pool IV and V) and one pool (VI) with non-wheat allergic control sera (IgE against grass pollen) diluted 1:25 were used. For reference, the cells were stimulated with a monoclonal anti-human IgE antibody (clone Le27-NBS01 mouse anti-human IgE-Fc Region Antibody; 500 ng/mL, NBS-C Bioscience, Vienne, Austria). All samples and reference release were corrected for spontaneous release in supernatants from unstimulated cells. The results are

expressed as the ratio of β-hexosaminidase release for samples on the release obtained for reference. The ratio calculated was considered positive if above 10%.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.02 for Windows software (La Jolla, CA, USA). Data were represented as the mean ± standard error of the mean. They were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant when p values were below 0.05.

3. RESULTS

3.1. Preparation and characterization of heated and hydrolysed gliadins

The process of heating gliadins resulted in a cloudy solution that was acidified with 0.2 M of acetic acid for 15 min prior to pepsin hydrolysis. The enzymatic hydrolysis of heated gliadins (HG and HGα) was performed under gentle stirring to maintain a homogeneous suspension at 37°C. The reaction was carried out for 60 min and several samples remained turbid.

The samples were characterized according to particle size by LLS experiments, and later the soluble fraction was analyzed by RP-HPLC.

3.1.1. Measurement of particle size in the suspension

The particle sizes of heated gliadins (HG) and heated and hydrolyzed gliadins (HhG) were determined by LLS (Fig. 1).

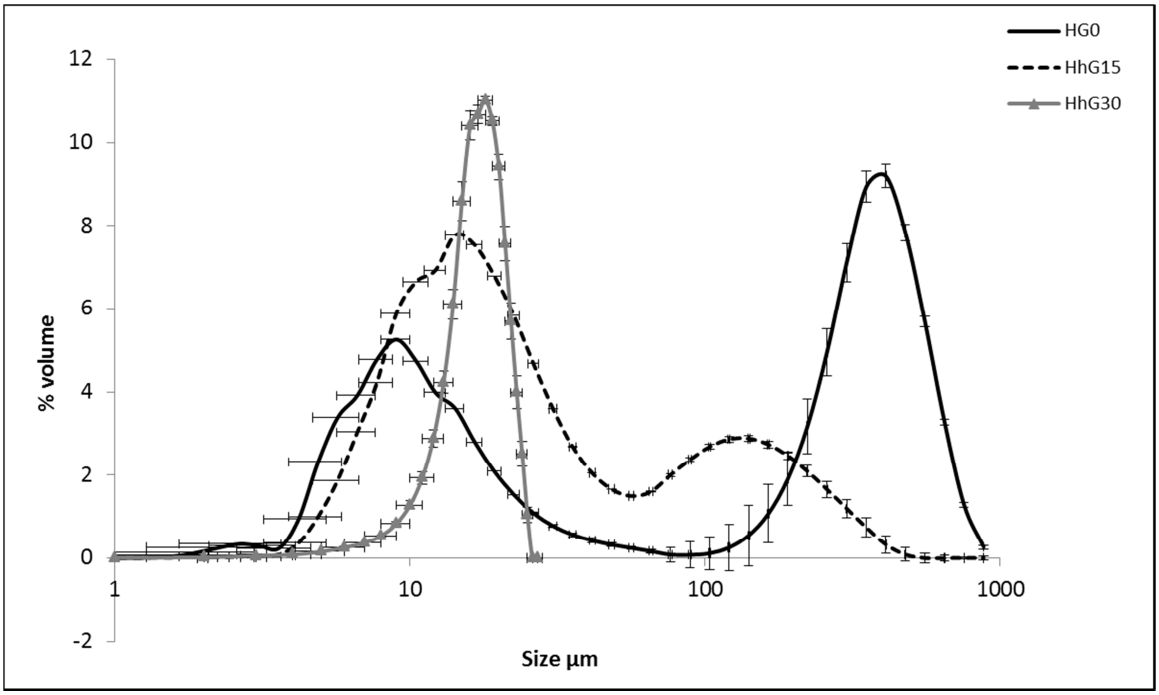


Fig. 1: Particle size distribution of heated gliadins (HG solid line), heated and hydrolyzed 15 min (HhG15 dotted line) and 30 min (HhG30 gray line).

Heated gliadins were characterized by a bimodal distribution of two particles with mean sizes of 9 µm±0.72 µm and 409 µm±1.3 µm. After 15 minutesof pepsin hydrolysis, this bimodal profile remained but was shifted towards the smaller size. The initially largest particles were reduced to smaller particles of approximately 140 µm ± 0.13 µm.

Additionally, another very broad peak covering particle diameters from 9 to 80 µm appeared with a maximum at 14 µm ± 0.14 µm, and a shoulder at approximately 10 µm can be seen. After 30 minutes of hydrolysis, particle size distribution became monomodal, with particle sizes of approximately 11 µm ±0.01 µm.

3.1.2. Soluble protein analysis by RP HPLC

The composition of the soluble fractions was followed during the time course of the hydrolysis (Fig. 2a-b) and compared to the non-heated gliadins, GN and Gα. The peaks corresponding to native gliadins, GN and Gα, which eluted from 35 to 55 min and 40 to 50

min, respectively, were no longer visible after heating (HG and HGα), whereas for both samples, one single very broad and flat peak appeared. These peaks eluted between 17-45 and 20-40 min for HG and HGα, respectively. Pepsin hydrolysates of HG (HhG0, HhG15, HhG30) and HGα (HhGα0, HhGα5, HhGα30) exhibited profiles characterized by two peaks, one eluting very early (approximately 10 min) and a second very large main peak, comparable to that obtained for HG and HGα. It is noteworthy that the intensity of these peaks increased with hydrolysis time and that the second one moved progressively towards shorter elution times.

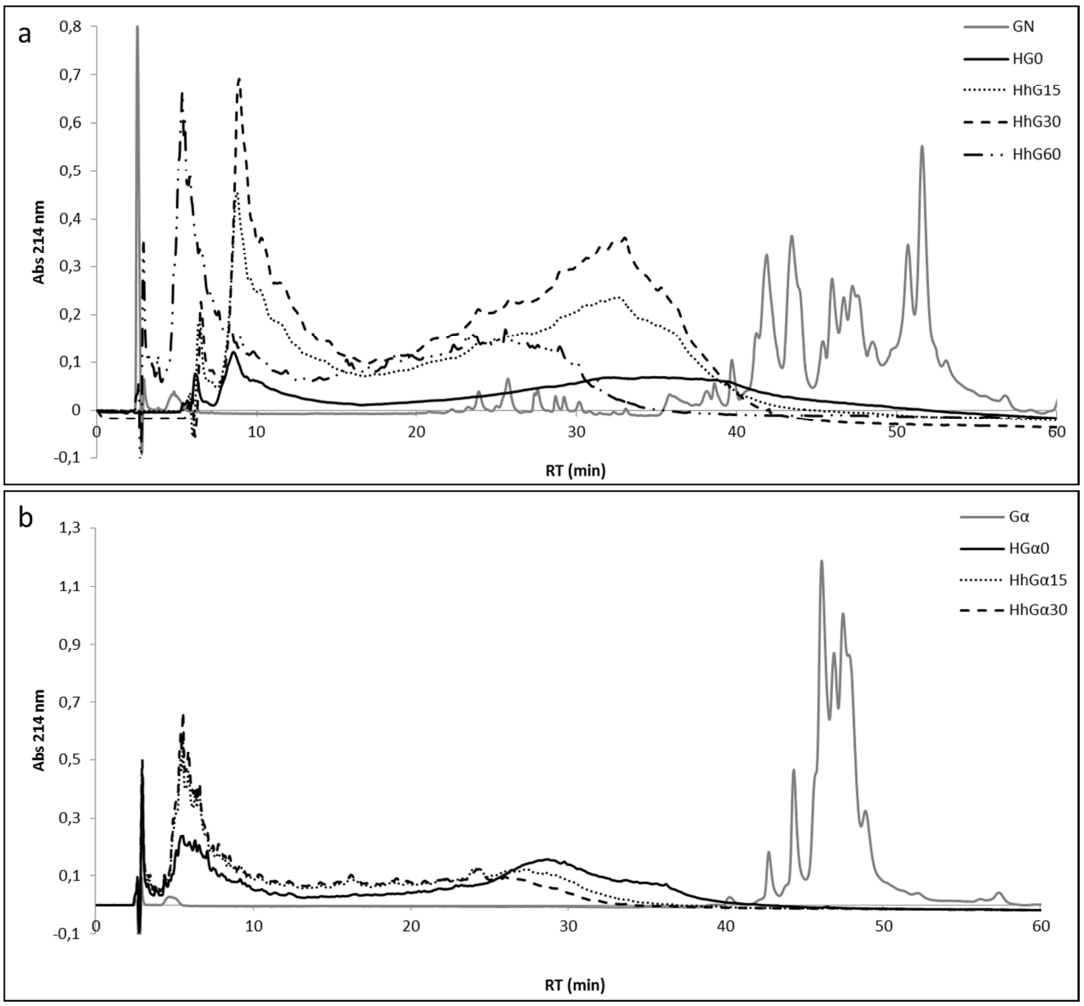


Fig. 2: RP-HPLC chromatograms corresponding to total gliadins (a), α-gliadins (b) and their heated-digested forms.

3.2. Immunogenicity and allergenicity of heated gliadins followed by gastric digestion

The antigenicity of gliadins after heating and hydrolysis was characterized with three antibodies specific to different gliadins domains (N-terminal, C-terminal or repetitive domain of gliadins) by Western blot. Any IgG epitopes were detected with immunoblots for Hh samples using N- and C-terminal antibodies, which revealed pepsin hydrolysis in these two regions. In contrast, the use of the anti-repetitive domain antibody revealed the persistence of immunoreactive polypeptides even after 30 min of pepsin digestion (results not shown). Allergenicity was tested with patient' sera pooled into groups according to their specific IgE reactivity against GN and Gα. Dot-blots were performed with three pools of patient sera, groups I, II, and III.. Increasing amounts of proteins were spotted on the membrane, ranging from 2 to 20 µg for native forms and from 10 to 60 µg for heated and digested samples. The three groups tested recognized both GN and Gα, the latter to a lesser extent. Since pool II and III gave very similar dot blot patterns, only the results obtained with pools I and III are presented in Fig 3.

No spots were revealed for the heated products (HG0 or HGα0) on the blotting membrane with any sera. IgE binding occurred again only after a certain duration of pepsin hydrolysis. In the cases of pools I and III, 5 minutes of gastric digestion were sufficient for recovering the recognition, whereas 10 extra minutes were needed in the case of group II. The three pools showed the highest reactivity against HhG30 and the lowest for HhG5. Only pools I and III recognized the products after 60 min of hydrolysis (HhG60). Regardless of sera group, α-gliadins were less recognized than GN. In the cases of pools I and II, the same reactivity was observed for HhGα15 and HhGα30. Low IgE-binding was observed for pool III in the case of α-gliadin samples. The dot blot revealed that heating and digestion masked some epitopes, but some persisted in the heated and digested forms after up to 60 min of hydrolysis.

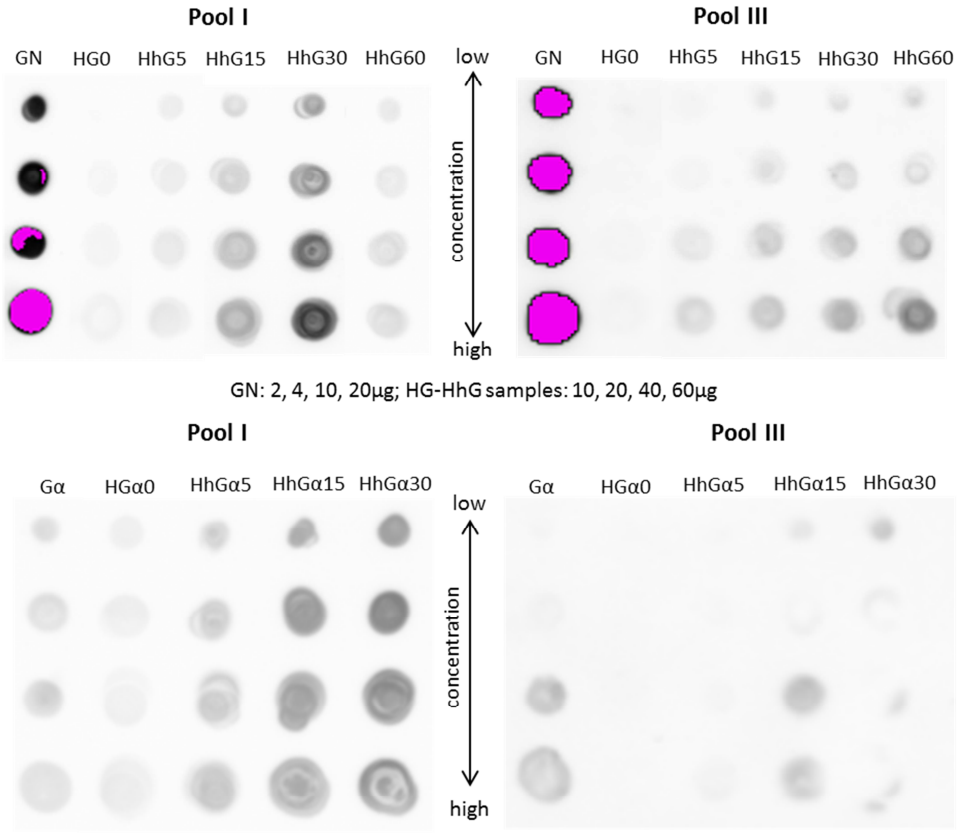


Fig.3: Dot blot IgE pattern against gliadins and α-gliadins, heated and digested forms with 2 pools of allergic patient sera.

3.3. Gliadin transepithelial transport and processing across Caco-2 cell monolayers

No significant change was observed in the viability of Caco2 cells after a 24 h exposure period to native, heated and digested total gliadins (results not shown). To evaluate monolayer integrity and to reduce growth variability, the TER value was checked after Caco-2 cell differentiation in the transwell plate.

The paracellular flux was monitored by the amount of AS-FITC marker in the basolateral medium and increased linearly for a 6 h period after sample deposition. The paracellular flux, measured by the slope of the marker in the basolateral medium, was significantly different between the deposited samples. The samples prepared from GN were not different from

controls without protein (C), and those prepared from Gα led to a significant increase in flux (Fig. 4A). Regardless of group, the paracellular flux was not affected by thermal or hydrolysis treatment.

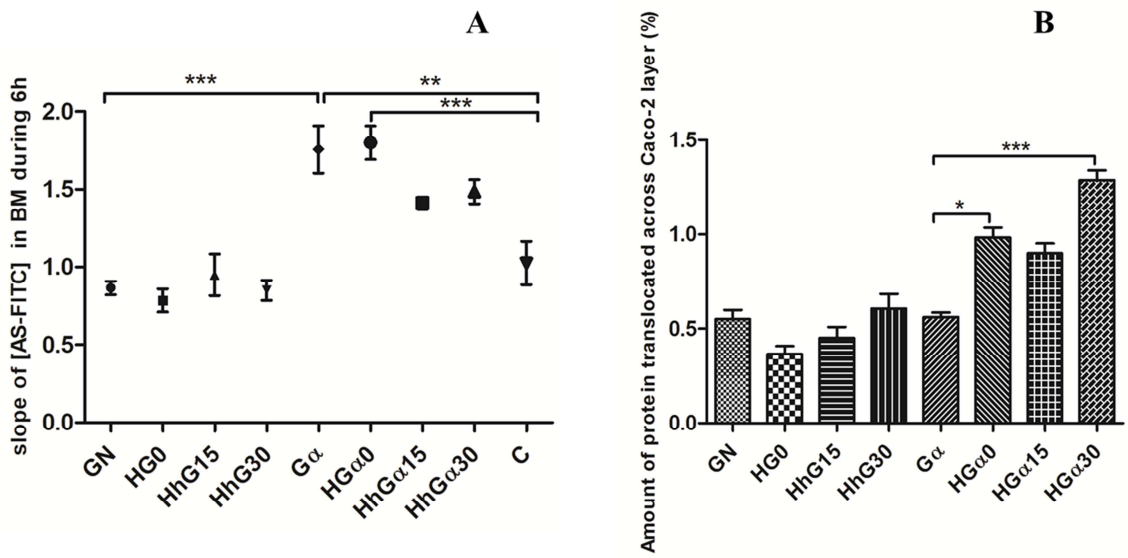


Fig. 4 A: Slope of AS-FITC concentration in basolateral medium during transport tests. C: corresponds to AS-FITC transport without proteins. **B**: Quantification of wheat proteins translocated across Caco-2 insert performed by ELISA with polyclonal antibody (PQQPYPQQPC). The results were expressed as the ratio of quantified proteins on basal and apical compounds. Significant differences are drawn; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

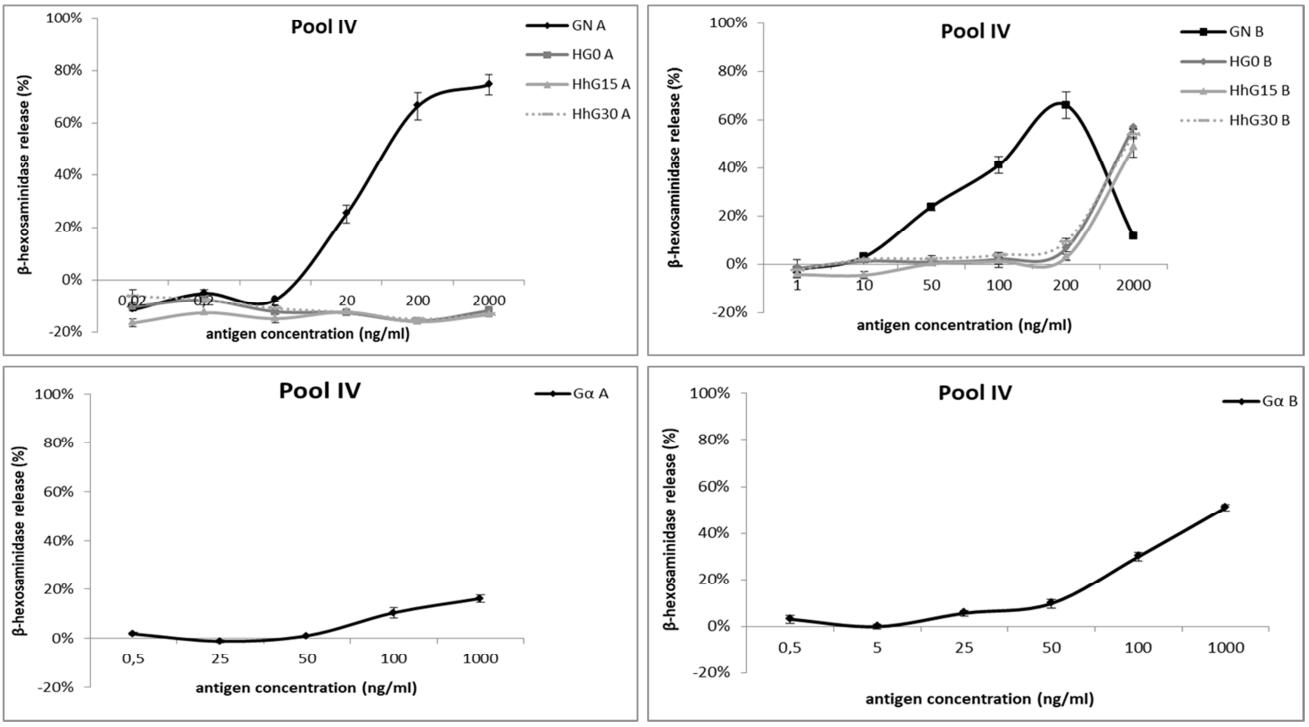
The capacity of native and modified (H and Hh) proteins to migrate across the monolayer was investigated by inhibition ELISA test measuring the amount of proteins in both the apical and basolateral compartments. The amount of proteins translocated from apical to basal media is expressed as the % of gliadins initially quantified in the apical compartment as reported in Fig. 4B. Approximatively 0.6% of the GN or Gα native samples were able to cross the Caco-2 monolayer. In the case of GN, no difference in the amount of translocated proteins was obtained after processing. The opposite situation was observed for Gα, for which the H and Hh forms were more translocated. A significant increase of the translocation was observed for HGα0 and HhGα30, with respect to their native forms.

3.4. Degranulation in vitro test

The triggering capacity of peptides transported across the Caco-2 monolayer was examined by the RBL-SX38 model using two pools of allergic patient sera (pools IV and V), both characterized by a slightly higher amount of IgE against GN than against Gα.

Pools IV and V were used for GN, HG0-HhG30, Gα and HGα0-HhGα30 samples. Pool VI was used as a negative control with basal solutions obtained from native and modified gliadins, and as expected no degranulation was measured (Fig. S1).

A



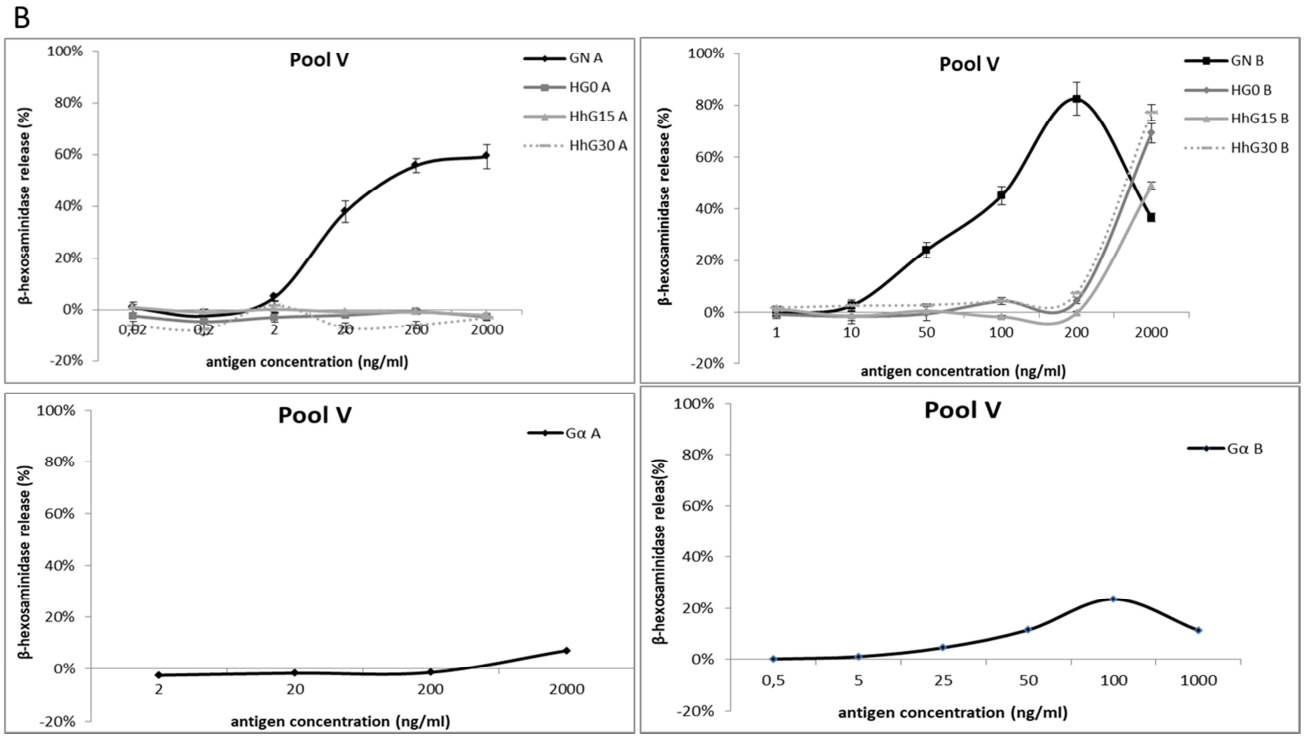


Fig.5: Basophil activation test with two representative pool of sera from patients allergic to wheat). ; pool IV (A) and pool V (B). RBL-SX38 cells were stimulated with GN, HG0, HhG15 HhG30, Gα, from apical and basolateral compounds.

The native, heated and heated/digested samples were evaluated via the RBL assay before and after incubation in the apical cell compartment and after the crossing the Caco-2 monolayer in the basal compartment (Figs. 5A-B and S2).

Before crossing the epithelial cells, the GN form was able to trigger β-hexosaminidase release for RBL cells sensitized to both pools, but none of the processed forms (HG0, HhG15 HhG30). Compared to gliadin analysis after their solubilization in the buffer (Fig. S2), the 6 hour incubation of gliadins on the apical side of the Caco-2 cells did not change the biological activity (Fig. 5). Before and after incubation in Caco-2 apical media, no activity was observed for the heated and heated/digested gliadins.

After Caco-2 cell crossing, the processed samples recovered an activity. Though the GN form still displayed better capacity to trigger β-hexosaminidase release with a MaxD at 200 ng/ml, whereas the H and Hh samples were only active at a higher concentration, 2000 ng/ml. A

control sample (basal cell media obtained after 6 hours without protein) did not induce cell degranulation (Fig. 5).

The Gα incubated in Caco-2 media (apical compartment) did not induce degranulation of RBL cells sensitized with the two pools but after transport across Caco-2 cells, we observed a degranulation by up to 51% of degranulation for the same protein concentration pool IV and 24% for pool V .

4. DISCUSSION

In many cases, allergy symptoms occur quickly after food ingestion. In the study by Makela et al., approximately half of the children with a positive oral food challenge to wheat reacted with immediate symptoms (i.e., within 2 hours) (Makela et al., 2014). Clinical case reports (https://www.allergyvigilance.org) (Czaja-Bulsa & Bulsa, 2017) show that reactions can occur within minutes of wheat ingestions. However, it is likely that undigested or weakly digested proteins may be involved in symptom elicitation. Baking impacts wheat digestibility, antigenicity of gluten proteins (Pasini et al., 2001; Petitot et al., 2009; Simonato et al., 2001; Smith et al., 2015) and allergenicity. Nevertheless, data on the capacity of baked and digested allergens to trigger the immune system are scarce and lacking in the case of wheat allergens. The main reason for this lack of data is likely because the use of complex food matrices such as bread or pasta is incompatible with assays on cellular models; that is why we chose to simplify the system and use purified gliadin fractions (total and α-type) alone. To understand better the allergen epithelial crossing and triggering capacity, gliadins were subjected to thermal treatment and pepsin digestion to obtain different forms that may be present in the intestinal lumen. Products of each step of this process were considered as potential luminal allergens and were easier to handle and more compatible with the *in vitro* cellular models used. The sizes of particles in suspension and abundance of soluble polypeptides were

characterized, and they were compared for their ability to cross intestinal epithelial cells and trigger effector cells.

Heating gliadins induced the formation of two populations of macroscopic aggregates, and only a few gliadins remained in solution. The early and quick decrease of the size of large aggregates after pepsin hydrolysis may be more related to the acidity of the reaction medium than to the pepsin action. Nevertheless, the persistence of aggregates around 200 µm after 15 min of hydrolysis reflects the strong ability of gliadins to interact through hydrophobic interactions and to self-organize into micelle type aggregates, as described by Herrera et al. (Herrera, Veuthey, & Dodero, 2016). The disappearance of the largest aggregates after 30 min of pepsin hydrolysis concomitantly with the increase of the soluble peptide fraction indicates that the cleavage of peptide bonds by pepsin contributes to the destabilization of these aggregates. The presence of particles after 30 min of hydrolysis reveals that the aggregation of the gliadins limits the accessibility of pepsin. This effect on the accessibility of the sequence is very clear on the repeated domain, which is still recognized by specific antibodies, whereas the N and C terminus domains are no longer recognized. The repeated domain, because of its repetitive structure and amino acid composition, is more resistant to hydrolysis than the flanking domains, and this resistance persists after heating. Smith et al. (Smith et al., 2015) also found a reduction of the accessibility of proteins and starch to digestive enzymes with heating and a matrix effect. Our results obtained using a simplified model of wheat proteins showed similar effects at the protein level. All substrates, native, heated and digested were further studied. We considered them to be likely present in the digestive tract during the digestion phase and therefore potentially able to reach the immune system. A proportion of wheat IgE-mediated allergies are triggered by gliadins whose IgE-recognized epitopes are predominantly linear and mostly localized in the repetitive domain of α/β, γ and ω types

(Battais et al., 2005; Denery-Papini et al., 2011; Matsuo et al., 2004). This domain also contains most T cell epitopes involved in celiac disease (Sollid et al., 2012). In the specific case of α-gliadins, heating at 85°C of the N-terminus repeated domain of the molecule caused an irreversible denaturation, which is not the case for other repeated domains, such as that of ω gliadin, which remained stable (Mameri et al., 2012). In the case of γ and α gliadins, the C-terminus non-repetitive domains contain cysteine residues. They also include some epitopic residues, since the disruption of disulfide bounds causes a reduction of IgE binding capacity (Mameri et al., 2015). After heating, the gliadins (total or α-type fraction) are present as aggregates, which were no longer recognized by IgE from patient sera. At this step, no epitope remained accessible, as shown by our dot blot assay or by the RBL activation test carried out when proteins were solubilized before their incubation in Caco-2 cell apical media (Fig. S2).

Gliadins, like other dietary proteins, undergo thermic treatments before being ingested and digested in the GI (gastrointestinal) tract. The roles of large polypeptides (more than 3000 Da) generated by digestion and their IgE binding capacity have already been reported (van Beresteijn et al., 1995; Moreno 2007; Bodinier et al., 2007). These fragments are likely to cross the intestinal epithelial barrier and interact with the immune system. The digestion of heated gliadins led to a reduction in the size of aggregates, and at the same time the protein chains were cut enzymatically. The digests thus released epitopes of IgG and IgE types. These immunologically active forms are likely to reach the level of the intestinal mucosa that we mimicked using human intestinal epithelial cells (Caco-2). This simple cellular model can express many properties of small intestine, including apical microvilli, tight junctions, and carrier-mediated transport systems (Cubells-Baeza et al., 2016).

Digested proteins (gliadins and α-gliadins) obtained after heating maintained their antigenicity due to persistence or unmasking of a few epitopes available for IgE antibody-binding. Nevertheless, the number of accessible epitopes may not be sufficient for biological activity because these proteins did not display eliciting activity, as shown in the RBL-SX38 test with samples (before incubation on apical compartment of Caco-2 epithelium cells). Incubation with the Caco-2 cell apical side where proteins might be affected by brush border enzymes did not alter the results. The inefficient hydrolysis of human and Caco-2 intestinal brush-border membrane enzymes on some gliadins peptides among them the 25-mer was already shown (Iacomino et al., 2013; Mamone et al., 2007). According to previous studies, we confirmed that only a low percentage of proteins is transported in the basolateral chamber (Stuknytė et al., 2015; Bodinier et al., 2007) due to a the efficient barrier function of the Caco-2 cells. Differences in the paracellular permeability induced by total gliadins or α-type gliadins may result from the complexity of the total gliadins fraction, which includes molecules of different sequences and structures. The increase of paracellular permeability in the presence of α-gliadins could be related to the capacity of some α-gliadins peptides to bind to a chemokine receptor present on the Caco-2 cell line that is involved in tight junction impairment (Lammers et al., 2008). In addition, although several gliadins and glutenins peptides have been identified as T-cell epitopes, the 33-mer peptide released from α2-gliadin is considered the predominant immunogenic peptide (Ciccocioppo et al., 2005), whereas other peptides, such as p31-43 from α-gliadin, are involved in the mucosal inflammation mechanism (Caputo et al., 2012). These epitopes are able to trigger the CD immune reaction and are present in the repetitive domain (Moreno et al., 2016; De Vincenzi et al., 2010; Lammers et al., 2015). In the case of total gliadins, the absence of the paracellular permeability effect may be related to the presence of other types of gliadins, including γ-, ω- and β, which did not impact the Caco-2 permeability (Bodinier et al., 2007). The number of

α-gliadins within the total gliadin fraction is not sufficient to affect paracellular permeability. Nevertheless, GN shows the best IgE binding capacity and basophil activation (before and after crossing the epithelial barrier). This is probably also due to other gliadin sub-fractions (β-, γ- ω-types). Nonetheless, even if the repetitive domain of the α-gliadins is involved in both CD and food wheat allergies, the epitopes involved differ, and in the case of FAW, additional IgE epitopes are located in the variable domain of α-gliadins. We assume that the lack of degranulation capacity of modified α-gliadins before and after crossing the Caco2 monolayer is probably due to the alteration of the α-gliadins structure by heating (Mameri et al., 2012) whereas other gliadin sub-fraction maintain active epitope structures after heating (Mameri et al., 2012).

Moreover, we observed the ability of gliadins even in their aggregated forms to cross the Caco-2 cell monolayer which is not known to promote the passage of aggregates. Indeed, using *in vivo* mice model with β-lactoglobulin (BLG) (Roth-Walder 2008) highlighted a preferential pathway according to the forms of this protein: native forms transcytosed through enterocytes while its aggregates were uptaken by Peyers's patches. This preferential pathway was confirmed in an *in vivo* model where the crosslinked BLG and Ara h1 (Price et al., 2014) were, like the native proteins transported through polarized Caco-2 cells. In the case of BLG, aggregation caused a reduction in the amount of translocated proteins (Stojadinovic et al., 2014). Rytkonen et al., using *in vitro* cell models observed that native BLG was more efficiently transported by M cells than by Caco-2 cells, whereas no differences between Caco-2 and M cells in the transport of heat-denatured BLG was determined (Rytkonen et al., 2006). Our study quantifies the transport of aggregated gliadins through Caco-2 monolayer and moreover shows that the crossed forms have an ability to trigger RBL cells. Because the H and Hh forms are unable to induce degranulation even after 6 h in the apical compartment of

the Caco-2 cell monolayer and also because the forms present in the transwell basal compartment are able to induce basophil degranulation, we can surmise that translocation across the Caco-2 monolayer induces an unmasking of some epitopes, probably due to cell-processing (Ménard et al., 2012; Ménard & Heyman, 2010). This phenomenon might also be due to a combined action of the brush-border membrane (BBM)-associated proteases and peptidases, which are expressed and active in these types of cells (Howell et al., 1992; Picariello et al., 2015), and of endosomal enzymes.

In our study, we confirm that aggregated forms of gliadins are able to cross the Caco-2 epithelial monolayer, endocytosis could be involved in gliadin transport and processing, as hypothesized in the case of Ara h1 and Gly m1 (Price et al., 2014; Sewekow et al., 2012).

5. CONCLUSION

Our results provide increased knowledge on the digestive process of some molecular allergens, their IgE-binding capacities, the resistance of some peptides to hydrolysis and their capacity to induce an allergic reaction. Finally, food processing leads to impaired IgE recognition in specific analytical methods such as ELISA or dot blot, but does not drastically reduce the allergenicity of gliadins, thanks to the subsequent action of pepsin and epithelial cells.

The presentation of the antigen in the food determines its future in the gastrointestinal track. Moreover, gastrointestinal digestion has been studied in Infogest Cost Action, especially with the establishment of guidelines for digestion steps, but other data are needed in the context of allergies, such as data on the role of BBM. This paper also emphasizes the importance of epithelial cell processing, and its key role in eliciting the capacity of food allergens needs to be further explored.

We suggest that the association between Caco-2 cells and the RBL-SX38 cell degranulation test is an interesting model to study the effects of heating and digestion on allergens. In the frame of risk assessment this tool Caco-2/RBL could be very useful to test and evaluate residual allergenicity of allergens after processing.

Conflict of interest

A part of the results were presented in poster P86 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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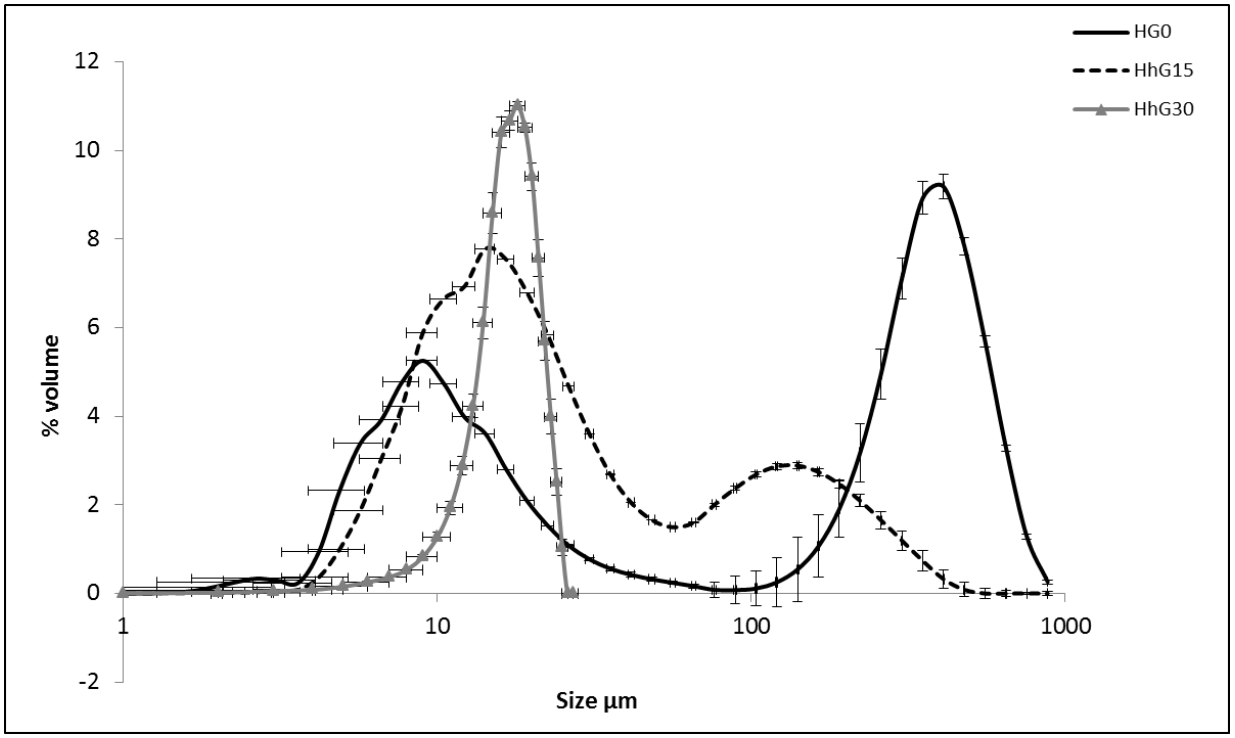


Fig. 1

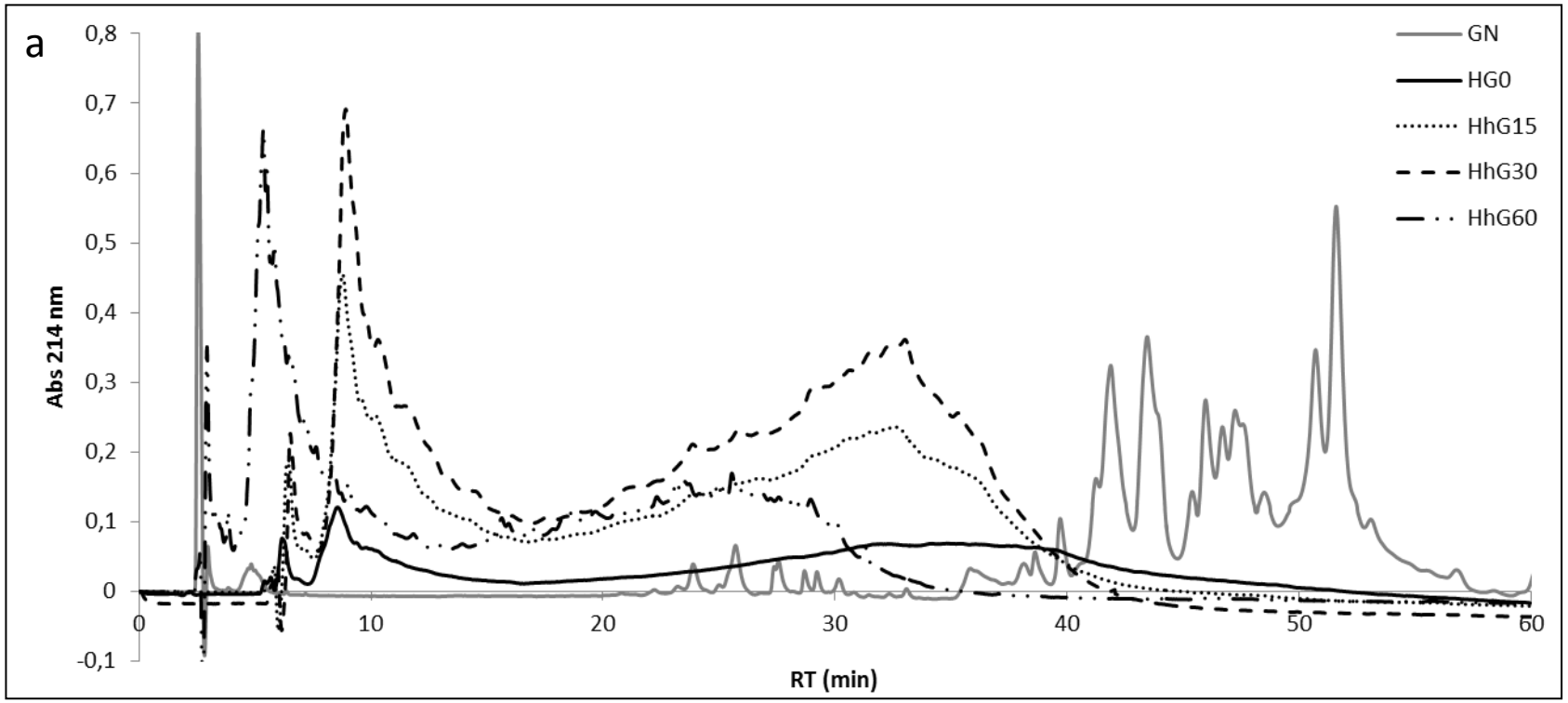

a
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
Abs 214 nm
0
10
20
30
40
50
60
RT (min)
GN
HG0
HhG15
HhG30
HhG60

Fig. 2a

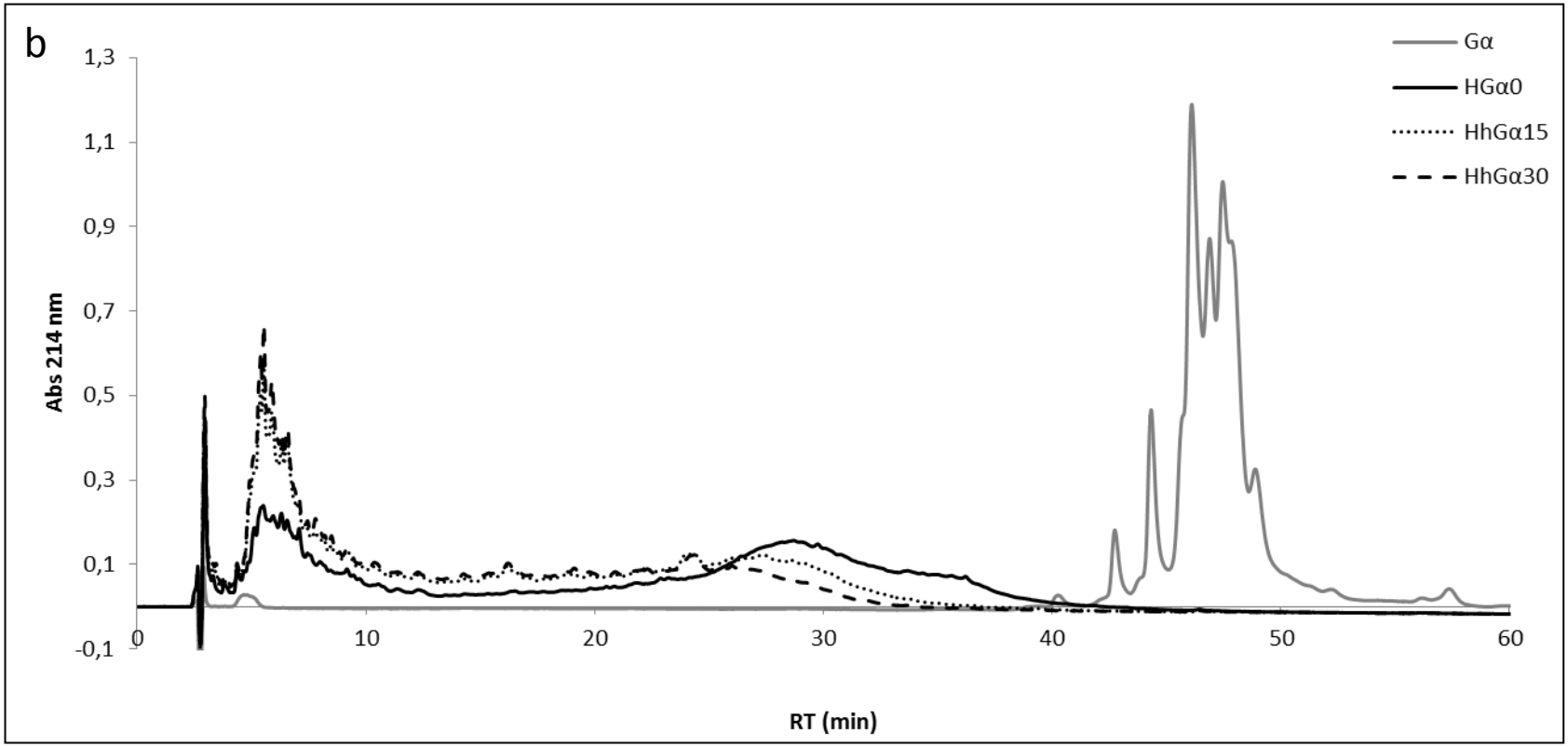
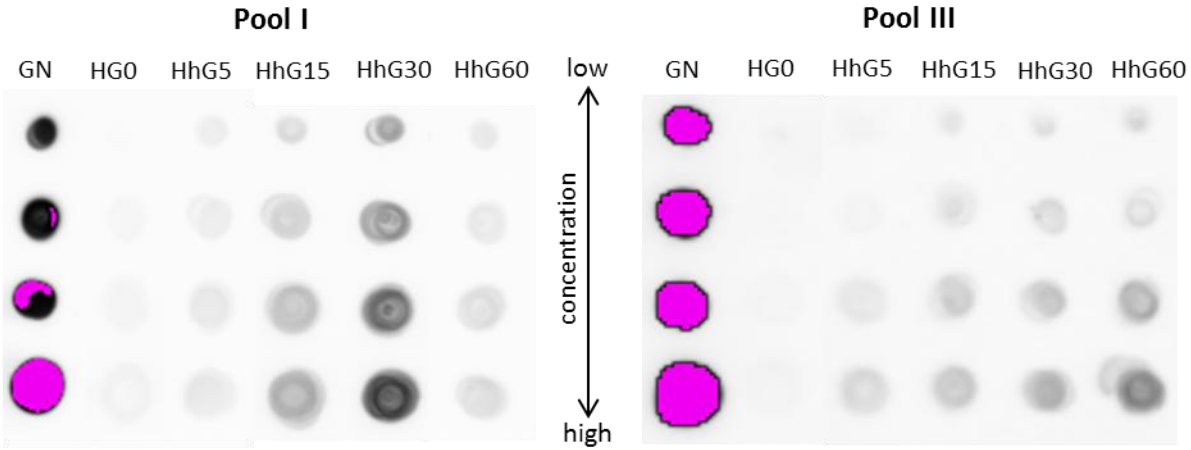
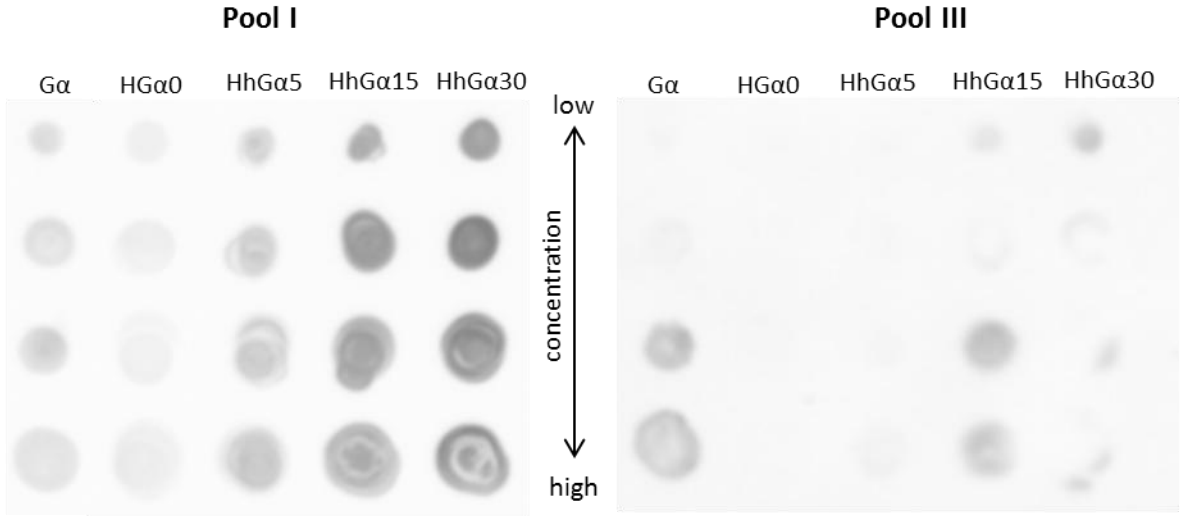

b
Gα
HGα0
HhGα15
HhGα30
Abs 214 nm
1,3
1,1
0,9
0,7
0,5
0,3
0,1
-0,1
0
10
20
30
40
50
60
RT (min)

Fig. 2b



GN: 2, 4, 10, 20μg; HG-HhG samples: 10, 20, 40, 60μg



Gα: 2, 4, 10, 20μg; HGα-HhGα samples: 10, 20, 40, 60μg

Fig.3

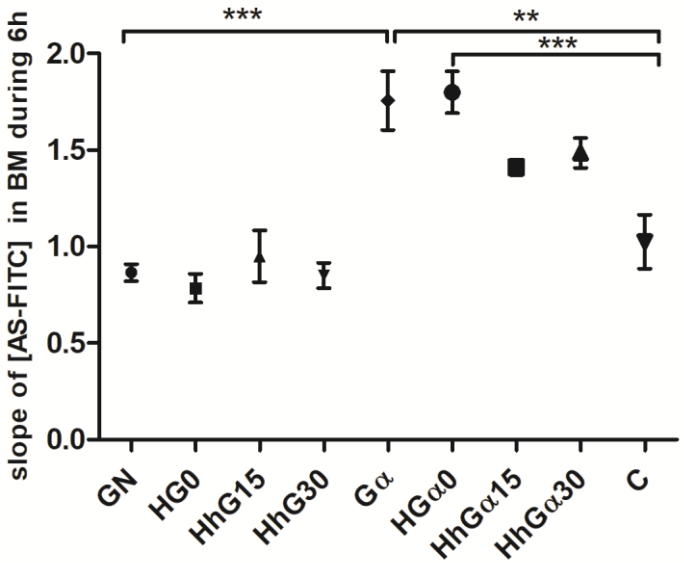

slope of [AS-FITC] in BM during 6h
2.0
1.5
1.0
0.5
0.0
***
**
***
GN
HG0
HhG15
HhG30
Gα
HGα0
HhGα15
HhGα30
C

Fig. 4a

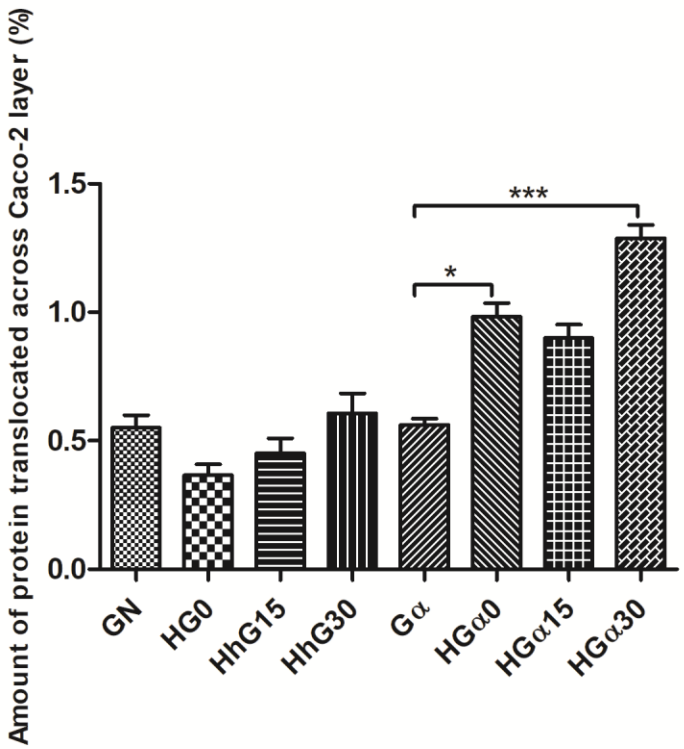
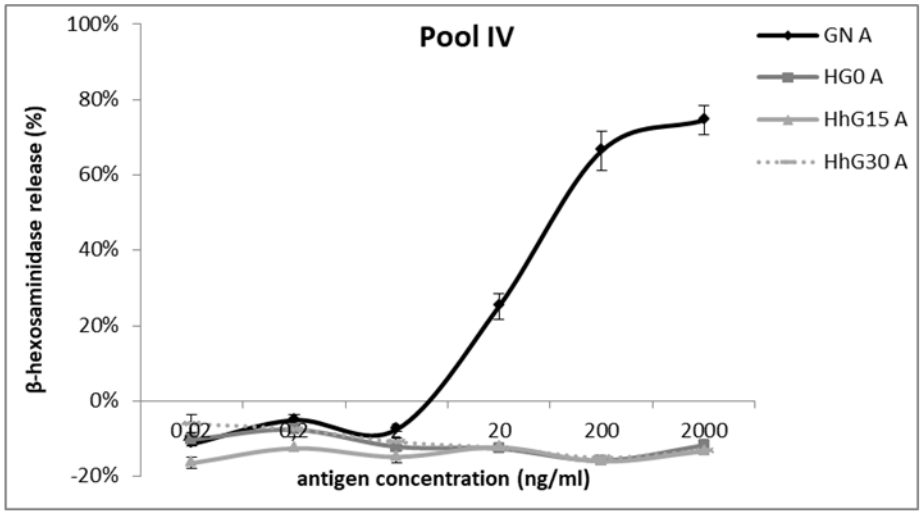
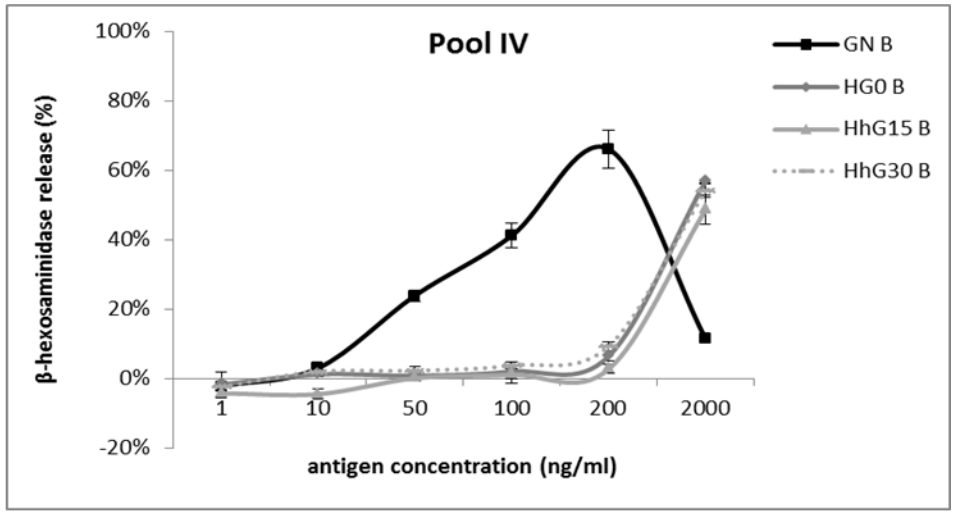
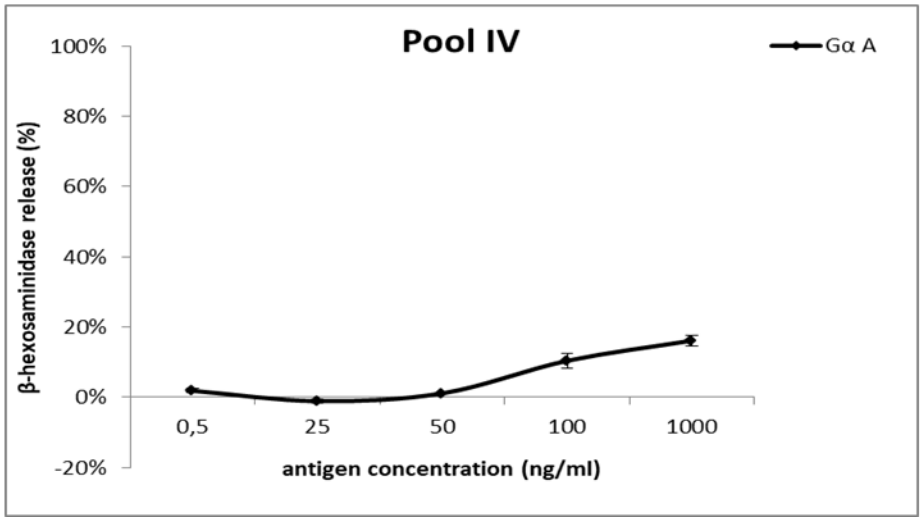

Amount of protein translocated across Caco-2 layer (%)
1.5
1.0
0.5
0.0
***
*
GN
HG0
HhG15
HhG30
Gα
HGα0
HGα15
HGα30

Fig. 4b

A


Pool IV
GN A
HG0 A
HhG15 A
HhG30 A
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
0,02
0,2
2
20
200
2000
antigen concentration (ng/ml)


Pool IV
GN B
HG0 B
HhG15 B
HhG30 B
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
1
10
50
100
200
2000
antigen concentration (ng/ml)


Pool IV
Gα A
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
0,5
25
50
100
1000
antigen concentration (ng/ml)

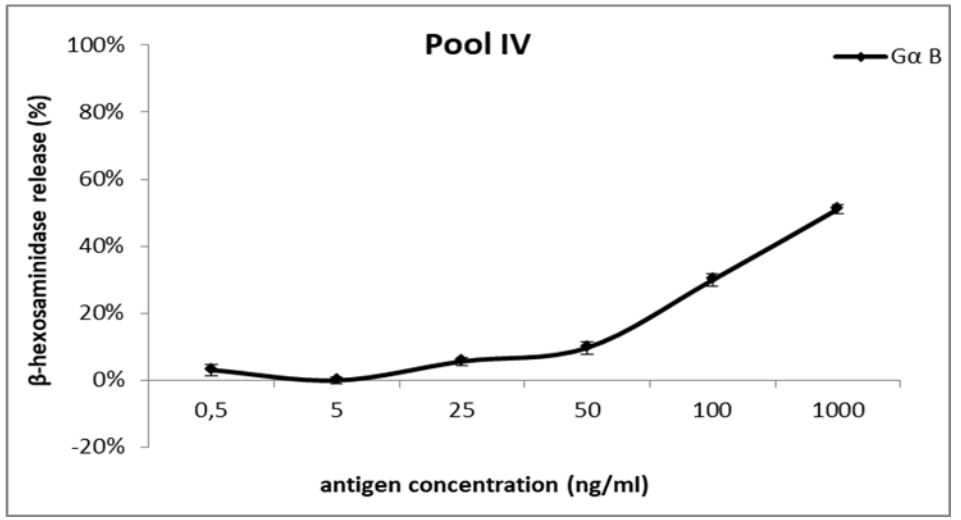
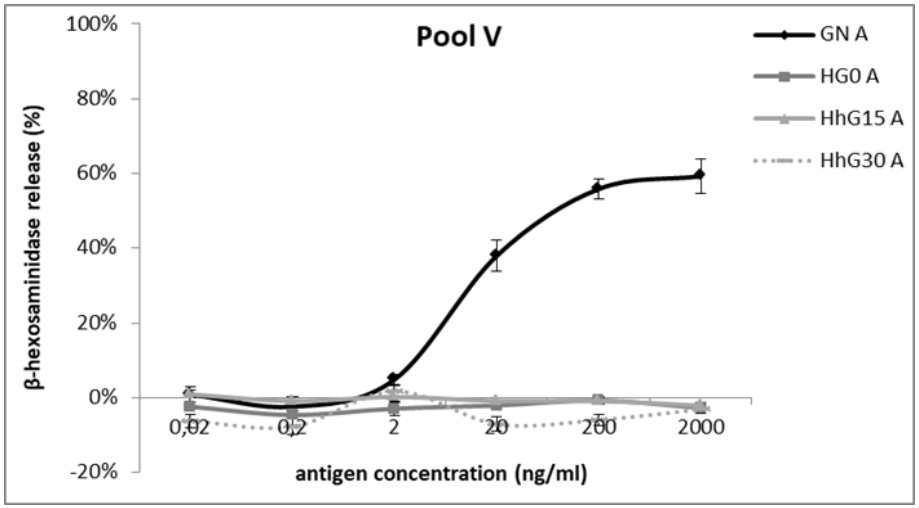
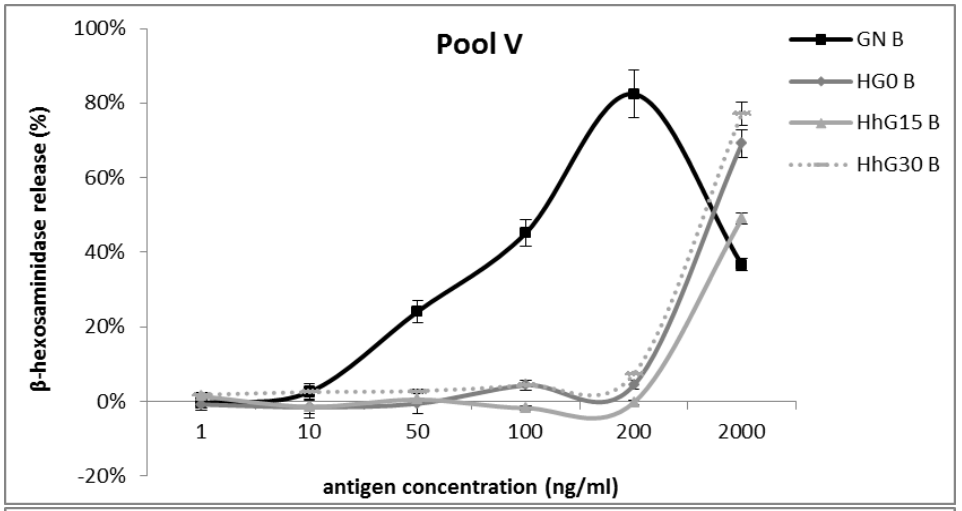
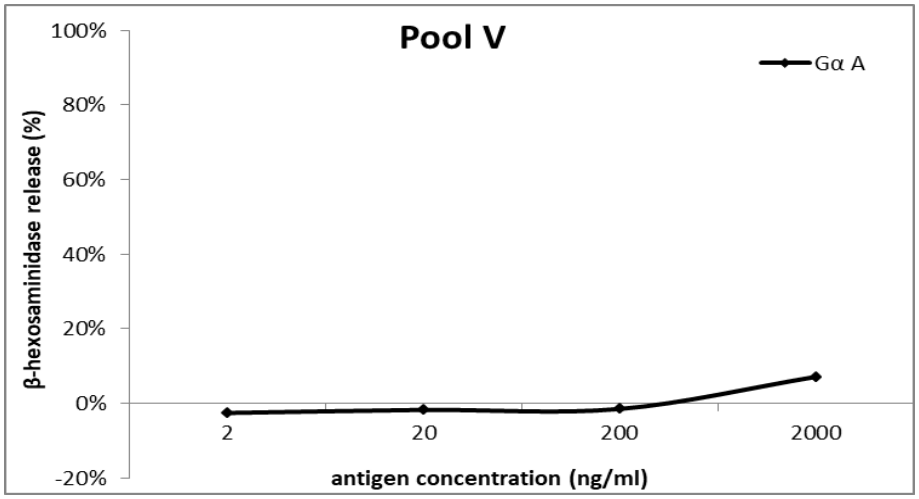

Pool IV
Gα B
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
0,5
5
25
50
100
1000
antigen concentration (ng/ml)

Fig. 5 A

B


Pool V
GN A
HG0 A
HhG15 A
HhG30 A
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
0,02
0,2
2
20
200
2000
antigen concentration (ng/ml)


Pool V
GN B
HG0 B
HhG15 B
HhG30 B
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
1
10
50
100
200
2000
antigen concentration (ng/ml)


Pool V
Gα A
β-hexosaminidase release (%)
100%
80%
60%
40%
20%
0%
-20%
2
20
200
2000
antigen concentration (ng/ml)

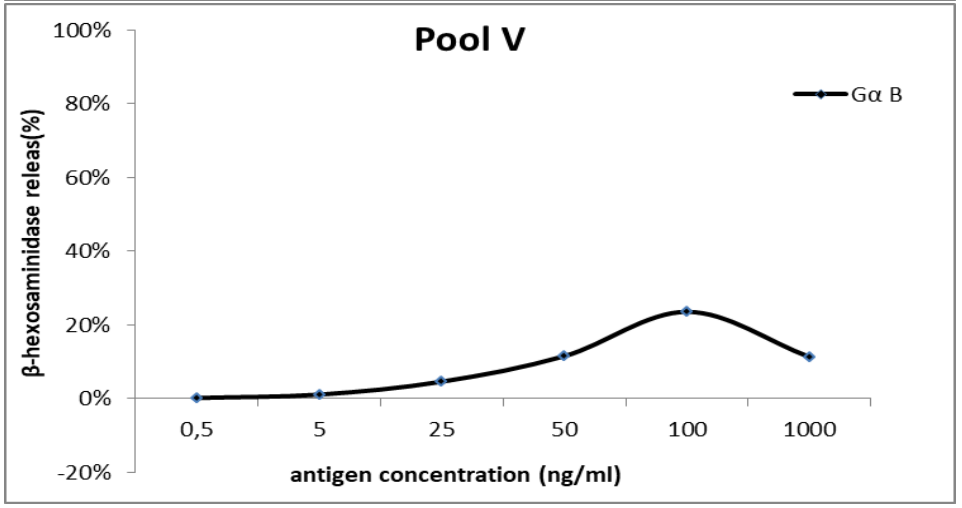

Pool V
Gα B
β-hexosaminidase releas(%)
100%
80%
60%
40%
20%
0%
-20%
0,5
5
25
50
100
1000
antigen concentration (ng/ml)

Fig. 5 B