Assessing the Effects of Processing on IgE Binding

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It is recognized that the IgE binding capacity of a protein or of a whole food is not a good predictor of its clinical reactivity, i.e. its ability to elicit an allergic reaction in sensitized individuals. Although IgE binding studies have a poor specificity and sensitivity for assessing the allergenicity, they are commonly used for the evaluation of the safety of new foods. They have also been also recommended in the guidance documents for the safety assessment of novel foods and particularly foods derived from biotechnology published by FAO/WHO (2001), Codex Alimentarius (2003) and EFSA (2004). They have provided useful information on the molecular mechanisms of initial steps of the allergic reaction and on the relationship between structural features and immunoreactivity/allergenicity. They also allow comparisons between raw *vs* processed or foods (e.g. in which proteins may be present under their native or denatured/modified structure). They are thus extensively performed to get at least indications on the effects of the processing on allergenicity and are used as a tool for a better management of the allergenic risk of foods, including for labeling purposes of products derived of common allergic foods.

Actually food processes, e.g. thermal treatment or fermentation, are used for increasing food qualities in terms of safety and storage ability by inactivation of microbes and toxins, in nutritional value through improvement of digestibility and in pleasure through improvements of appearance, texture, flavour and taste. The indirect relationship between food processing and allergenicity is based on a rationale which is apparently quite simple. Heat treatments or proteolysis during the fermentation alter the structure of proteins, i.e. the allergenic constituents of a food and, as a consequence, alter their allergenic potential and therefore the allergenicity of the whole food. Although no clear and general relationship between the structure of a protein and its allergenicity has been established, this view assumes, albeit not explicitly, that allergenicity is an intrinsic property of a protein due to or strictly related to particular structural features.

Significant alterations in protein structure do occur during heat treatments, the nature and extent of such changes being dependent on the temperature and duration of the thermal processing as well as on the intrinsic characteristics of the protein and the physico-chemical conditions of its environment (e.g. pH, presence or absence of water …). Typically loss of tertiary structure is followed by (reversible) unfolding, loss of secondary structure (55-70°C), cleavage of disulphide bonds (70-80°C), formation of new intra-/inter-molecular interactions, rearrangements of disulphide bonds (80-90°C) and the formation of aggregates (90-100°C). These modifications reflect a progressive passage to a disorganized structure with denaturation of the proteins that adopt an unfolded, random-coil conformation. The denatured molecules associate to form aggregates and then gels resulting in a modification of the surface properties and an increase in size. Besides those physical transformations, chemical modifications of the protein may also occur at high temperatures (100-125°C and higher). These may involve formation of covalent bonds between the lysine residues of a protein and other constituents of the food matrix leading to various adducts. Advanced glycation end products, carboxymethyl lysine, malondialdehyde and 4-hydroxynonenal formed through protein-sugar interactions (i.e. by the Maillard reaction) and the cross-linking of oxidised lipid products with proteins are commonly found and may contribute to the formation of new immunologically reactive structures (Davis, 1998; Besler, 2001; Chung, 2001).

During proteolysis, e.g. by pepsin or trypsin, the protein is split and several peptide fragments are formed, the nature of which depends on the amino acid sequence of the protein but also on its secondary structure (e.g. presence of disulfide bonds) and on the post translational modifications (e.g. glycosylation or phosphorylation patterns, point mutations) the protein has undergone. The peptides may then re-associate to form agglomerates.

However, the assumption that any modification of its native conformation would alter the allergenicity of a protein and consequently of the whole food cannot be considered a general evidence. It does not take into account the qualitative and quantitative variability of the allergen repertoire of a whole food which includes several proteins sometimes under different isoforms, the multiplicity of conformational and/or linear epitopes on a given allergen or the genetic/geographic variability of the immune response in atopic humans. In addition, interactions with other constituents of the food matrix may occur and have a major effect on the overall allergenicity of the food. The effects of every food processing on allergenicity/IgE binding of a complex food may therefore very likely vary in an unpredictable way depending

on the process itself, on those different factors and conditions and on their interactions. Those rearrangements occurring during processing may result in either destruction of existing epitopes, particularly conformational epitopes, or unmasking of existing epitopes that were hidden in hydrophobic regions within the tertiary structure of the native protein molecule and creation of new ones which IgE binding studies permit to analyse.

This variability in the effects of thermal treatments has been observed by many authors. Pastorello (2003) did not observe any loss of IgE-binding capacity in a lipid transfer protein (LTP) of maize, after a thermal treatment at 100°C for 160 minutes. Wigotzki (2001) also showed that dry processing at 100°C for up to 90 min had no effect on the allergenicity of some hazelnut proteins and suggested the occurrence of very heat-stable allergenic proteins with molecular weight less than 14 kDa. However, the IgE-binding capacity of other hazelnut allergens was decreased after 15-minute heat treatment at a temperature between 100 and 185°C. According to Hansen (2003), roasting for 40 min at 140°C led to a decrease in allergenicity of the birch pollen-related allergens Cor a 1.04 and Cor a 2 but increased the allergenicity of the LTP Cor a 8. Heat treatments can destroy conformational epitopes and result in the fact that only linear epitopes are still available for binding to IgE. Denaturation may thus explain the loss of 90% of immunoreactivity of heat-labile birch pollen-related allergens of hazelnuts such as Cor a 1.04 and Cor a 2. This is of major importance for the management of hazelnut allergy in people of Central and Northern Europe mainly sensitised to Bet v 1 related allergens or in people of Southern Europe who are mainly sensitised to LTP (Hansen, 2003).

Methodology

IgE binding studies can be performed using different techniques. It is noteworthy that the same kind of tests may be used for 2 different purposes:

1. To detect or confirm the diagnostic of a specific allergy/sensitization in a patient provided that a standard well characterized allergen is used as reference material.
2. To assess the allergenicity/IgE binding capacity of a compound which is related to or derived from a known allergen using the serum of relevant and well characterized patients whose allergy to the source (food or protein) is well established.

This presentation will essentially focus on the second aspect and on the use of ELISA derived immunoassays such as Enzyme Allergo Sorbent Assays (EAST) and EAST inhibition. Other techniques are also classically used such as western blot which associates 1-D or 2-D electophoretic separations of proteins and identification of the allergens by immunoblotting using sera of allergic patients. However a possible important bias is that electrophoresis are often performed in denaturing conditions, i.e. using sodium dodecyl sulphate polyacrylamide gel (SDS PAGE) under reducing conditions, which modifies the structure of the allergen(s). This may also be the case when EAST are performed using allergens immobilized on the solid phase (e.g. microtiter plates) which require care in the interpretation in terms of specificity and affinity of the allergen antibody intreraction.

Cell based tests such as the various tests based on the allergen induced degranulation of human basophils of sensitized individuals or of passively sensitized basophils or mast cells of human or animal origin are also a kind of IgE binding studies which give more accurate information on the biological relevance and functionality of the binding.

Briefly the principle of the most common format of EAST and EAST inhibition studies is the following:

1. Microtiter plates are coated with a whole protein aqueous extract of either the native or processed food or with purified proteins/allergens prepared from the raw food or from products derived thereof by processing or digestion,
2. After washings, saturation of the plates, washings, serial dilutions of sera of patients whose allergy to the (native) food has been recently confirmed by clinical investigations (clinical history, skin prick testing and possibly oral food challenges) are dispensed in the microtiter plate,
3. Plates are incubated in appropriate conditions (temperature, time, agitation …) then extensively washed
4. An anti human IgE antibody, usually a monoclonal antibody, labeled with an enzymatic tracer is added. After incubation the plates are extensively washed, then
5. The substrate of the enzyme is added and the colorimetric/fluorimetric reaction allowed to develop.

Results are generally expressed in terms of absorbance and one difficulty of EAST is the absolute quantification as ng or IU of specific IgE bound to the allergen.

In order to confirm the results of direct EAST studies and to compare the IgE binding capacity of different products, EAST inhibition tests are generally performed. The same procedure as that described above is used except that a preliminary reaction is added before step 2, i.e. allergic patient sera are first pre-incubated with inhibitor solutions. Inhibitors are the different processed products or digesta. After incubation, the serum/inhibitor mix is dispensed on a microtitre plate coated with the standard allergen of reference. The procedure then continues at step 3 and followings.

Results are expressed as $B/B_{0,}$ where B_0 and B correspond to the specific IgE binding to immobilized standard allergen in the absence or presence of a known concentration of inhibitor, respectively. Inhibition curves are plotted and inhibition concentrations needed to inhibit 50% of the IgE binding to coated allergen (IC50) are compared between the different samples. The higher the 50% inhibition concentrations, the lower the residual immunoreactive potency of the sample.

The performance of this kind of study depends on the quality of the reagents (e.g. second antibody, enzymatic tracer, standard allergens) and particularly on the purity of the extracts and purified allergens and derivatives in order to minimize false negative and false positive results. However the characteristics and relevance of the sera used are of major importance. Their heterogeneity in terms of IgE concentrations, specificity and affinity of those IgE is an important source of variability. In order not to miss any information it is preferable to perform the test with individual sera and not with pools of sera where the weight of a particular serum of a highly sensitive individual might be big and mask the reaction of other ones.

Besides this natural variability and heterogeneity of individual IgE responses which may result in a large dispersion of the results, some other characteristics of the individual immune response of the patients might be a source of pitfalls. As an example an immune response with an important production of high affinity specific antibodies of IgG subclasses may interfere on the IgE binding because of a competition between IgEs and IgGs for the coated allergen. Although the immobilized allergen should in principle be present at a large excess,

the removal of serum IgG using for example Protein G may then be necessary to avoid an underestimation of the IgE binding.

In order to increase the specificity and sensitivity of the competition, other formats have been developed. An alternative consists of coating anti human IgE on the titer plate instead of the allergen, then to incubate the allergic sera in order to first bind the antibodies of the IgE class and discard the unbound IgGs. In a further step, after extensive washings, the reference allergen that has previously been labeled, is added and incubated in presence or absence of increasing concentrations of inhibitors.

This will now be illustrated by experimental studies on the effects of thermal treatments and of digestion on the IgE binding capacity of peanut and milk allergens.

I. Influence of thermal processing on the IgE binding of peanut proteins

Peanut allergy is one of the most common and severe IgE-mediated reactions to food because of its severity and lifelong persistence (Sampson, 2004). The prevalence of peanut allergy has been estimated between 0.6 and 1% of the US and EU population and seems to have increased during the last decade (Sicherer 2002, 2003). In contrast, sensitisation and reactivity to peanut is far less prevalent in China, despite the high rate of peanut consumption. It was shown to be related to Chinese dietary habit to eat boiled peanuts and to the traditional method of cooking peanut in water which reduces their allergenicity as compared with roasting, which predominates in the US (Beyer, 2001).

Among the different peanut seed storage proteins, some are well characterized allergens, e.g. the 7S globulin Ara h 1 and the 2S albumin Ara h 2 (Burks, 1991, 1992). The aim of this study was to assess changes in the IgE-binding capacity of whole peanut proteins and of the 2 major peanut allergens Ara h 1 and Ara h 2 after thermal processing like roasting and boiling in comparison with raw peanut.

Thirty-seven children (mean age: 8 years; median: 5 years) were recruited at the paediatric allergy clinic of the Hopital Necker-Enfants Malades Paris, France. All the patients had a confirmed peanut allergy based on an extensive history, physical examination, skin prick testing and objective manifestations observed after peanut ingestion.

All the patient sera were individually tested.

Preparation and characterization of whole peanut protein extracts and purified allergens

Whole peanut protein extract (WPPE)

Raw and commercially roasted peanuts (Virginian variety) were used. Kernels of raw peanuts were boiled for 30 minutes in water. After cooking, the water (Water PE) was collected and used for further analysis of the protein content. After each treatment, peanut kernels were peeled and ground then defatted. Proteins were extracted several times by stirring in aqueous buffer then in 4 M urea. The pooled supernatants of the extracts from raw (Raw PE), roasted (Roasted PE) and boiled (Boiled PE) peanuts were then dialysed and stored at –80°C. An aliquot of the Raw PE was heat-treated by boiling for 30 minutes. Protein concentration in the extracts was determined using the BCA method (Pierce).

Purified allergens

Ara h 1 and Ara h 2 were prepared and purified from raw, roasted and boiled PE by precipitation using 40% ammonium sulphate. then separated and purified by a combination of chromatographic methods including i) affinity chromatography on a Con A Sepharose column, ii) RP HPLC on AKTA purifier system (Pharmacia) on a C4 column, elution: acetonitrile 0.04% TFA.

Ara h 2 was isolated from the unbound fraction of the Con A Sepharose affinity chromatography. This fraction was dialysed. After addition of 4 M urea, the dialysed fraction was first purified by anion exchange chromatography. Purification was then achieved using the same reversed phase chromatography as for the purification of Ara h 1.

Ara h 1 and Ara h 2 were characterized by electrophoresis, MALDI TOF mass spectrometry analysis and by N-terminal amino acid sequence analysis using the Edman method.

706 mg of protein were obtained from 5 g of raw and roasted peanut kernels. Only 377 mg of proteins were obtained from 5 g of boiled peanut kernels, e.g. a 2-fold lower recovery. Moreover, proteins were found in the water used to cook the peanuts. The protein

concentration in the water was 39 mg in 100 ml (i.e. volume used to cook 10 g of peanut kernels).

The protein compositions of Raw, Roasted, Boiled and Water PE were compared using SDS PAGE under reducing conditions at the same concentration (i.e. 1 $mg.ml^{-1}$). As shown in figure 1 A, similar patterns were observed in Raw PE, Roasted PE and Boiled PE including a band of ca. 65 kDa molecular weight (MW) corresponding to Ara h 1, several bands ranging from 25 to 45 kDa, which very likely correspond to glycinin fragments, and 2 bands at ca. 16 and 18 kDa corresponding to the 2 characteristic isoforms of Ara h 2. It is noteworthy that Ara h 1 and Ara h 2 concentrations in Boiled PE were much lower than in Raw and Roasted PE. In addition, low MW protein bands ranging between 10 and 16 kDa, which correspond to 2S albumins or peptide fragments (MW <10 kDa), were still present in Boiled PE, although in lower amounts than in Raw and Roasted PE. The cooking water electrophoretic pattern shows the presence of proteins and particularly of Ara h 2 and of those low MW proteins whose concentration was decreased in Boiled PE.

Figure 1B shows the corresponding western blot performed using one representative allergic serum. In all the extracts, the same allergens were present i.e. Ara h 1 (MW of ca. 65 kDa), glycinin fragments (MW ranging between 25 and 45 kDa), Ara h 2 (2 isoforms of MW of 16 and 18 kDa) and proteins of low MW ranging from 10 to 16 kDa very likely represented by 2S albumins and fragments thereof. In Boiled PE, the concentration of Ara h 1, Ara h 2 and other 2S albumin bands was lower than that observed in Raw and Roasted PE.

Purified and characterized Ara h 1 and Ara h 2 were recognized by specific IgE in the same manner regardless which of the three extracts (Raw, Roasted and Boiled PE) they were prepared from.

IgE binding studies on the whole peanut protein extracts

The IgE-binding capacity of the different extracts was analysed using an EAST adapted from Bernard (2003). Microtitre plates were coated with the different whole peanut protein extracts (i.e. Raw, Roasted, Boiled and Water PE) at a concentration of 10 $\mu g.ml^{-1}$. Serial dilutions of each serum were dispensed per well and incubated for 24 h at +4°C. An anti-human IgE antibody (BS17 clone) labelled with acetylcholinesterase (AChE) was used as a tracer. Ellman's reagent was used as enzyme substrate. Specific IgE were quantified by comparison

with concentration-response curves obtained with a total IgE assay performed under identical conditions using a solid phase coated with a second anti-human IgE antibody (LE 27) instead of peanut proteins, which is complementary to BS 17-AChE tracer according to Bernard (2003).

Figure 2 shows the IgE binding capacity of the different extracts as measured with each individual allergic serum. A marked heterogeneity was observed. Mean or median values for Raw and Roasted PE were very similar. Water PE had the lowest IgE binding capacity. Median value for Boiled PE was approximately 1.5- to 2-fold lower than those for Raw and Roasted PE. Interestingly, the sum of the median IgE binding values for Boiled and Water PE (Σ=181 IU.ml^{-1}) was close to those for Raw PE (144 IU.ml^{-1}) and Roasted PE (176 IU.ml^{-1}). No significant differences were observed between IgE binding of Raw and Roasted PE that were significantly higher than those of Boiled or of Water PE. Differences between IgE binding capacity of Boiled and Water PE were also significant.

EAST inhibitions were performed on Raw, Roasted, Boiled and Water PE as described above except that a preliminary step was added, i.e. 40 µl of allergic patient sera were first preincubated with 40 µl of inhibitor solutions (i.e. peanut protein extract) for 4 h at room temperature. Fifty microlitres of the serum/inhibitor mix were then dispensed per well on a microtitre plate coated with Raw PE. Inhibitor solutions contained increasing concentrations of Raw, Roasted, Boiled or Water PE (from 1 ng.ml^{-1} to 100 µg.ml^{-1}).

Results were expressed as $B/B_{0,}$ where B_0 and B correspond to the specific IgE binding to immobilised Raw PE in the absence or presence of a known concentration of inhibitor, respectively. IC50 were compared between the different samples for each individual allergic serum.

Figure 3 shows the inhibition curves of IgE binding to Raw PE obtained with one representative allergic serum by increasing concentrations of Raw, Roasted, Boiled and Water PE. The IgE-binding was partially or completely inhibited by each of the different peanut protein extracts and by the Raw PE that had been boiled for 30 min. Inhibition curves obtained with Raw and Roasted PE were similar. Boiled PE was a weaker competitor. IC50 values measured with Boiled PE were 10- to 50-fold higher than those measured with Raw

and Roasted PE. Interestingly, inhibitions obtained with the Raw PE that had been heat-treated (i.e. boiled) after extraction were very close to those obtained with Raw PE itself and higher than those obtained with Boiled PE. The protein extract from the cooking water gave parallel inhibition curves although the IC 50 was about 5-fold higher than with boiled PE. Similar results were obtained when Roasted PE was used as immobilized allergen instead of Raw PE.

IgE binding studies on the purified allergens

The IgE binding capacity of Ara h 1 and Ara h 2 purified from the different peanut extracts was analysed using the same sera. EAST inhibition studies were performed as described by Mondoulet (2005) On microtitre plates coated with a monoclonal anti-human IgE antibody (i.e. LE 27 clone), 100 microlitres of a dilution of each serum were distributed per well and incubated overnight at +4°C. After washing, 50 µl of inhibitor and 50 µl of tracer were dispensed and incubated for 4 h at RT. Inhibitors consisted of increasing concentrations of raw, roasted and boiled Ara h 1 or Ara h 2. Enzymatic tracers were prepared by covalent linkage of Ara h 1 or Ara h 2 to acetylcholinesterase (AChE). Colored reaction was developed and measured as above.

Increasing concentrations of purified raw, roasted and boiled Ara h 1 (respectively Ara h 2) were used to inhibit the binding of Ara h 1-AChE (respectively Ara h 2-AChE) tracers to immobilized allergic patient serum IgE. In both cases a complete inhibition was obtained with all the inhibitors. With all the patients, the highest inhibitions were observed with the allergen (i.e. Ara h 1 and Ara h 2) prepared from the roasted peanut as shown in figure 4. Specific anti-Ara h 1 and anti-Ara h 2 IgE of the patient whose serum was used to plot the inhibition curves in figure 4, were quantified and were 176 and 181 $IU.ml^{-1}$ respectively.

Discussion

The IgE-binding capacity of both the whole food, i.e. whole peanut proteins, and of purified Ara h 1 and Ara h 2 was analysed after roasting and boiling. Whole proteins, Ara h 1 and Ara h 2 were prepared from raw, roasted and boiled peanuts using the same procedure. The protein content was quantified then analysed. Ara h 1 and Ara h 2 had the same properties in terms of chromatographic and electrophoretic behaviour, N-terminal amino sequence and mass spectrometry. In particular, Ara h 2 isoforms were observed in Raw, Roasted and Boiled PE. Protein recovery was similar in Raw and Roasted PE. After boiling, part of the

protein was present in the Boiled PE but a part also remained in solution in the cooking water, particularly proteins of low MW.

In the study population, we observed a variability in IgE response to whole peanut protein extracts. However, the same heat processing effects on the IgE-binding capacity of whole peanut protein extracts were observed in all patients. The IgE responses to Raw and Roasted PE were not significantly different whereas those to Boiled and to Water PE were approximately 1.5- to 2-fold lower.

In addition to the quantification of specific IgE, the analysis of the effects of heat treatments on the allergenicity of whole peanut proteins by EAST inhibition gave similar information on the apparent affinity of the IgE-peanut protein interaction. Inhibition of IgE binding to immobilised Raw PE was similar when Raw PE or Roasted PE were used as competitors, but the IC50 of Boiled and Water PE were 10- to 50-fold higher than those of Raw and Roasted PE. The same observations were obtained when microtitre plates were coated either with Raw PE or with Roasted PE.

Inhibition studies on Ara h 1 and Ara h 2 were performed using an enzyme immunoassay different from the classical EAST. An inhibition test was developed to overcome difficulties in obtaining inhibition curves, particularly with Ara h 2, and to improve the sensitivity and specificity of the test. The competition was performed in solution between Ara h 1 and Ara h 2 purified from Raw, Roasted and Boiled PE and the same pure allergens labelled with AChE, for the binding to immobilized IgE. The difficulties were likely due to the structural polymorphism of Ara h 2 and to the heterogeneity of anti-Ara h 2 specific IgE populations. In this assay, all the IgE (including total IgE and IgE specific to peanut proteins) were immobilized using anti-human IgE monoclonal antibodies (LE27 clone) which were passively adsorbed on the microtitre plates. LE27 did not bind IgGs.

Regarding the effect of heat treatments for all the tested sera, Ara h 1 and Ara h 2 purified from Roasted PE showed a higher inhibitory capacity than those purified from Raw and Boiled PE. Interestingly, this difference was no longer apparent with whole food, i.e. whole peanut proteins.

Ara h 2 protects Ara h 1 from degradation by trypsin and this protective characteristic is enhanced by roasting (Maleki, 2003). An interaction with Ara h 2 during roasting treatment may explain the enhancement of the immunoreactivity of Ara h 1 from Roasted PE. Maleki et al. (2000) showed that the Maillard reaction products contribute to the increase in IgE-binding capacity of peanut after roasting. However, the increase in immunoreactivity of Ara h 1 from Roasted PE they observed was much greater than that observed in the present study. The discrepancy in Ara h 1 reactivity (and particularly in that of "roasted" Ara h 1) may be due to differences in the study population and particularly in the dietary habits of the children. Exposure to roasted peanuts may be greater for American than for European populations. It may facilitate and enhance sensitisation to the highly stable trimers of Ara h 1 formed during the roasting process.

In the case of Ara h 2, inhibition studies showed no difference between Ara h 2 from Raw or Boiled PE but a higher immunoreactivity after roasting. The structure of Ara h 2, a 2S albumin, has similar cysteine motifs as LTP and in consequence an LTP-like folding (Sellers, 2000) which results in the heat-stable property of those proteins (Schocker, 2000). Intramolecular cross-linking caused by roasting and the enhanced trypsin-inhibitory activity very likely contribute to the increase of allergenicity (Maleki, 2003).

The allergenicity of a whole food depends on the contribution of several allergens that may react differently to processing and to heat treatments. The nature, intensity, length and conditions of heat treatment may also impact differently on the structure of allergenic proteins, on their interactions with other constituents of the food matrix, and finally on their allergenicity. Some allergenic structures may be destroyed whereas others, particularly low-molecular-weight peptide fragments, may be newly formed. All those interactions may explain why the effects of heat treatments are attenuated or suppressed for whole peanut food as compared with isolated pure allergens.

The global decrease in allergenicity observed in boiled peanut was not associated with structural modifications of proteins but mainly with a loss of allergens, essentially of low-molecular-weight proteins or peptide fragments from kernels and their transfer by solubilisation into the cooking water with which they are discarded. This decrease of the exposure to peanut allergens and particularly to low MW allergenic fragments seems to

correlate with the low prevalence of peanut allergy in countries where peanuts are cooked in this way.

Wet process heating (e.g. boiling) instead of dry process could be used by the food industry to decrease the allergenicity of peanut protein fractions used for the preparation of different foods. However, such a partial decrease of the allergenicity might very likely not be sufficient to protect a vast majority of consumers allergic to peanut and would not be an appropriate measure for the management and prevention of the allergy risk. Because of the uncertainty on how much allergenicity may remain after processing and of the uncertainty on the threshold doses of proteins or immunoreactive fragments that may elicit reactions in sensitive people, the labelling of processed food products prepared from allergenic foods like peanuts is essential for the information of allergic consumers.

II. Influence of thermal processing on the IgE binding of milk proteins

Using the same methodology, the IgE binding capacity of major milk allergens, e.g. □ lactoglobulin and whole casein fraction, was studied on the purified proteins prepared either from raw defatted milk or from boiled milk. As shown in figure 5, once again the effect of thermal treatment differs depending on the protein. Casein IgE binding capacity is not affected while □ lactoglobulin IC 50 is increased (ca. x5) which means that the allergenicity is reduced but not abolished. Those observations are in line with what is known on the structure of those proteins.

Whole casein fraction comprises 4 different proteins, i.e. αS_1-, αS_2-, β- and κ-casein. Each individual casein represents a well-defined chemical compound but they cross-link to form ordered aggregates (i.e. micelles) in suspension in lactoserum (whey). The 4 caseins have little primary structural homology but they display common features that are quite unusual, differing greatly from other milk proteins. The most important caseins, i.e. □- and □-caseins have a dipolar-type structure, comprising a globular hydrophobic domain and a highly solvated and charged domain, with amphipatic properties. They are phosphorylated proteins, with a loose tertiary, highly hydrated structure (Schmidt, 1982). Whole casein is often considered poorly immunogenic because of this flexible, non-compact structure and because it is rapidly and extensively degraded by proteolytic enzyme during digestion. As confirmed

by this study, casein structure and function are not significantly affected by severe heat treatments and the allergenicity remains unchanged as well.

On the opposite lactoglobulin is a globular protein, with the classical ß barrel structure of lipocalins comprising arrangements of 8 antiparallel β sheets, stabilized by 2 disulfide bonds (Papiz, 1986 ; Flower, 1996). It is quite resistant to degradation by proteases but is thermo labile.

As a consequence, heat processing has no definite nor unequivocal impact on cow's milk or dairy products allergenicity. Formation of aggregates may increase the allergenicity of the heated product. When the treatment results in a decrease of the allergenicity, it is always limited. As reported in several studies, boiling of milk for a few minutes (2, 5 or 10 min) results either in no difference or in a reduction of about 50-66% of the positive reactions as compared to raw milk. Similar observations have also been reported with raw *vs* pasteurized or homogenised and pasteurized milk (Gjesing, 1986; Host and Samuelsson, 1988; Norgaard 1996; Werfel., 1997).

III. Influence of digestion on the IgE binding of peanut proteins, e.g. Ara h1

Gastro-intestinal sensitization is likely for many food allergens for which resistance to pepsinolysis has been considered to be a prerequisite. This has lead to resistance to pepsinolysis being used as a predictive tool or at least as an indication in the weight of evidence approach to assessing the allergenicity of novel proteins destined for food use (FAO/WHO, 2001 ; Codex Alimentarius, 2003 ; EFSA, 2004)), although later studies have indicated that the relationship between resistance to digestion and allergenicity may be less clear cut than was originally thought (Fu, 2002). To date there have been few studies on the effect of proteolysis due to digestion or to fermentation process on the allergenicty of proteins and complex foods nor on the possible role of digestion products of proteins in terms of sensitization and maintenance of food allergy. Recent studies performed on food allergens provided evidence that peptides resulting from an extensive digestion are capable of IgE-binding and/or T-cell activation (Hong, 1999 ; Diaz-Perales, 2003).

In its native form, one of the major peanut allergens, Ara h1 is a stable homotrimeric protein. The monomer-monomer contact points are the regions where the majority of the identified

IgE-binding epitopes are located (Maleki, 2000). In the case of crude peanut extracts it was assumed that no residual IgE binding capacity remains after gastric digestion (Hong, 1999) although Maleki (2000) reported digestion fragments with IgE reactivity. However, in most studies, immunoreactivity was detected with poly- or monoclonal antibodies raised against an intact allergen, rather than with specific IgE from peanut allergic patients. Moreover, denaturing conditions were applied for gel electrophoretic characterization, making it impossible to investigate the possibility that digestion may unmask epitopes or result in the formation of self-assembled peptide complexes. This might have resulted in some discrepancy between the different authors.

The effect of the digestion process on the allergenic properties (e.g. IgE binding capacity) of Ara h1 and of the resulting breakdown products was studied by applying a two step *in vitro* digestion protocol simulating gastric and duodenal *in vivo* digestion. This was a collaborative study performed within the EU funded programme ALLERGEST in which the *in vitro* digestibility test has been coupled with a battery of immunological measures of the allergenic activity of these fragments. In particular, studies were based on the whole IgE-repertoire in human sera from peanut allergic individuals with a convincing history of peanut anaphylaxis to assess the IgE-binding capacity.

Purified Ara h1 was prepared from hexane defatted peanut meal as previously described in section I. In vitro gastric and gastro-intestinal digestions were performed according to Moreno (2005 a and b).

The analysis were performed using each serum individually. The binding of specific IgE to whole peanut extracts and to purified Ara h1 was determined by EAST and EAST inhibition performed as described in section I.

***In vitro* gastric digestion**

Ara h1 (2.5mg/ml in 0.15M NaCl adjusted to a pH 2.5) was incubated with a solution of pepsin in a shaking incubator at 37^{o}C. Aliquots of digesta were regularly collected until 120 minutes digestion and kept into 0.5M ammonium bicarbonate solution to raise the pH to 6.8-7 and irreversibly inactivate pepsin. In order to simulate gastric digestion, pepsinolysis was performed in the presence or absence of egg L-phosphatidylcholine (PC). Digesta were analysed for protein composition by SDS PAGE under reducing conditions.

During gastric digestion intact Ara h1 was rapidly degraded with a loss of the M_r 74,500 polypeptide which was accompanied by the appearance of a series of fragments of M_r 6-19,000 together with very low molecular weight material. The bands were also rapidly digested, with only a broad band around M_r 6,000 remaining after 8 min digestion, while at the same time lower molecular weight polypeptides were formed, which remained stable for 2 h gastric digestion (Figure 6 A).

Competitive inhibition curves plotted using the serum of a representative patient are presented in Figure 6 B. These curves clearly demonstrate that increasing concentrations of the digestion products inhibit the binding of patients IgE to immobilized Ara h1. Gastric digestion did not alter the IgE-binding capacity of Ara h1 whether the digestion was performed in the presence or in the absence of (PC).

In vitro duodenal digestion

Duodenal digestion was performed using the gastric digesta as starting material. The pH was adjusted to a pH of 6.5 and the followings were added: a bile salt mixture containing equimolar quantities of sodium taurocholate and glycodeoxycholic acid; 1 M $CaCl_2$; porcine pancreatic lipase and porcine colipase; trypsin and α-chymotrypsin at approximately physiological ratios of Ara h1:trypsin:chymotrypsin=1:0.0025:0.01 (w: w). The digestion was performed at 37°C in this duodenal digestion mix. Aliquots were taken at various time points and the digestion was stopped by adding a solution of Bowman-Birk trypsin-chymotrypsin inhibitor from soybean.

The simulated duodenal digestion resulted in a very rapid loss of any polypeptides remaining following gastric digestion (Figure 7 A).

As shown on figure 7 B, gastro-duodenal digestion decreased the IgE reactivity of Ara h1 but the digesta were still capable of extensively inhibiting the binding of IgE to Ara h1. Again, the presence of PC did not affect the IgE reactivity of the gastro-duodenal digestion products. No effect was observed with negative controls indicating that the residual amount of enzyme present in the samples did not interfere with the antigen-antibody reaction during the EAST inhibition test.

For each serum, the IC50 values were calculated and confirmed the general trend as presented in the inhibition curves but showed the great heterogeneity in the individual patient IgE responses to Ara h1 and to its gastric/gastro-duodenal digesta. This reflects differences both in terms of specificity and affinity to patients IgE, suggesting a multiplicity of epitopes and different sensitization patterns in each individual. It is also related to the variability in the specificity and affinity to the IgE and the presence of numerous epitopes that are present on Ara h1 and/or may be released during the digestion of the protein. Using gastric digesta only a slight increase in IC50, corresponding to a small decrease in immunoreactivity, was observed. Following gastro-duodenal digestion a minor but significant loss of IgE-reactivity was observed for all sera. This is reflected in the increase in IC50 values of 2-3-fold times higher in presence than in absence of digestive enzymes. However, it is important to mention that the extent in the reduction of the IgE reactivity was limited.

In conclusion digestion by pepsin had no or minor effects on IgE binding and despite being broken down to small peptides the Ara h1 IgE immunoreactivity was almost unchanged. The presence of PC did not significantly impair the digestive process as to affect the immmunoreactivity of the fragments.

Discussion

A rapid degradation of the intact major peanut allergen Ara h1 monomer was known to occur in the presence of digestive enzyme (Kopper, 2004). This study demonstrated that Ara h1 retained its allergenic properties after digestion. Whilst Ara h1 is broken down by digestion into low molecular weight polypeptides, these retain the IgE binding properties of the intact protein in an almost unaltered form. This confirms the observations by Shin et al. (1998) of the rapid release of fragments possessing IgE-binding epitopes upon hydrolysis of Ara h1 monomer with various digestive enzymes. We propose that some IgE-binding epitopes of Ara h1 recognized by patients´ sera are not exposed by the undigested Ara h1 monomer, whereas other IgE-binding epitopes are unmasked during the digestion process. We confirmed the persistence of IgE binding epitopes for Ara h1 after the gastro-duodenal digestion recognized by patients´ sera by performing EAST inhibition assays. Following gastro-duodenal digestion the IgE-immunoreactivity of Ara h1 was significantly reduced but not abrogated, IC50 of gastro-duodenal digesta being 2-3 times higher than those of intact Ara h1, whilst those obtained for gastric digesta were generally similar to the intact protein.

The gastro-intestinal route of sensitization is one of the possible ways of sensitization to peanut allergens (Hourihane, 1996 ; Lack, 2003 ; Peeters, 2004). However, resistance to digestion is classically considered to be mandatory for this process and stability of relevant food allergens was described to be either “complete”, “intermediate” or “not stable” with regard to their pepsin resistance (Astwood, 1996). However, the absolute correlation between allergenicity and resistance to pepsinolysis has been questioned (Fu, 2002).

Digestion of Ara h1, whilst expected to abrogate IgE-binding capacity (Hong, 1999), had a modest effect on IgE-reactivity. We postulate that the self-assembly of low molecular weight peptide fragments into higher molecular weight complexes is responsible for this phenomenon.

IV. Influence of digestion on the IgE binding of milk proteins, e.g. lactoglobulin

In western countries, cow’s milk allergy is the most common food allergy in infants under 2 years of age but it also occurs in adults (Norgard, 1996 ; Hill, 1999; Sampson, 1999). In the majority of cases, allergic reactions to cow’s milk proteins are thought to be IgE-mediated (Sampson, 2001).

The globular protein β-lactoglobulin (BLG) is present in the whey fraction of the milk of most mammals, but not in human milk. Native BLG occurs as a 36 kDa MW dimer at neutral pH comprising identical subunits. BLG belongs to the lipocalin superfamily which adopt a β-barrel structure with a retinol-binding calyx stabilized by two intra-molecular disulphide bonds (Papiz, 1986). Such structural features are thought to contribute to the stability of this protein to, for example, proteolysis (Reddy, 1988). The relative resistance of BLG to acid hydrolysis as well as to proteases may allow some of the protein to escape gastrointestinal digestion. This increases the probability that intact BLG will be absorbed through the gut mucosa and may explain why it is one of the most potent allergens in cow’s milk.

The aim of the present study which was a collaborative study performed within the EU funded programme ALLERGEST was to investigate the effect of gastrointestinal digestion on BLG allergenicity and particularly on its IgE binding capacity.

In vitro gastric and gastro-intestinal digestions were performed on purified BLG according to Moreno (2005 a and b) as described in section III.

Residual amounts of intact BLG were quantified using the sandwich ELISA test described by Negroni (1998).

Twenty patients aged 7 months to 7 years (mean age 2.4 yrs) with a confirmed allergy to cow's milk were recruited at the Kyriakou Children's Hospital, Athens. Each serum was analysed for specific IgE titer. Sera with a sufficient amount of specific anti BLG IgE were used individually for EAST and EAST inhibition studies as described above.

***In vitro* gastric and gastro intetinal digestion**

SDS-PAGE analysis of gastric digestion products showed the presence of a single protein band of M_r 18,500 corresponding to that of intact native BLG even after 2h gastric digestion.

During subsequent duodenal digestion for 15 min the protein was broken down into a series of lower M_r polypeptides although some intact protein still remained.

The amount of intact BLG remaining in digesta were analysed with an ELISA specific for intact native protein. There was a slight reduction in intact BLG in all gastric digesta, with around 40% of the protein apparently being broken down. However, following gastro-duodenal digestion only around 3% of intact BLG remained, although the inclusion of PC in the gastric digestion appeared to completely protect the protein from degradation in the duodenal environment for at least 15 min. (see data on figure 8).

Figure 8 shows typical EAST inhibition curves obtained with sera from a single cow's milk allergic child. Gastric digestion had no effect on IgE binding, the same 50% binding inhibition doses (IC 50) of ca. 5 ng/ml being obtained for digesta and control incubations performed in the absence of proteases, for all sera analysed. For gastro intestinal digestion in the absence of PC no inhibition of binding was observed at the concentrations employed (Figure 8) indicating that the fragments present in the digesta had no residual IgE binding capacity. In contrast gastro-duodenal digestion in the presence of PC did not alter the IC 50 values which were 5-10 ng/ml for all the digesta, including the controls.

Discussion

As described before, BLG was found to be almost completely resistant to breakdown in the gastric compartment, reflecting its resistance to pepsinolysis (Schmidt, 1995). However, the

presence of the physiological surfactant PC in the gastric phase of digestion was found to protect the BLG from breakdown in the duodenal environment, in marked contrast to the susceptibility of the protein to trypsin and chymotrypsin in absence of PC. These data indicate that BLG is resistant to both gastric and duodenal digestion when physiologically relevant levels of PC are included in the digestion mix. The mechanism underlying the protective effect of PC on BLG breakdown in the duodenal environment is not clear but may be related to its ability to interact with lipids via its specific binding site (Godovac-Zimmermann, 1987 ; Reddy, 1988 ; Moreno, 2005). Using human cow's milk allergic sera the residual IgE binding capacity of BLG after digestion was found to be consistent with the levels of residual native protein found in the digesta.

This resistance and the presence of intact BLG in the digesta may of course explain its allergenicity. However, other studies have been performed on the effects of hydrolysis on BLG structure and allergenicity. Sélo et al. (1998) performed chemical hydrolysis of either native BLG or denatured BLG (i.e. after reduction and carboxymethylation which means a complete loss of the secondary structure) with cyanogen bromide which splits the proteins in 4 fragments. They showed that no residual intact BLG could be detected but that the IgE binding capacity remained intact in the CNBr hydrolysates. The same authors (Sélo, 1999) performed tryptic hydrolysis of BLG and found an IgE binding capacity in the hydrolysates and in some of the isolated and purified tryptic peptides although no intact BLG remained. They thus confirmed previous observations by Haddad (1979) who showed that specific IgE from 10 patients with cow's milk allergy recognised enzymatic digestion products of BLG by pepsin or pepsin + trypsin (10 patients out of 10) and that the recognition of peptides was even better than that of the intact molecule in 4 patients out of 10.

Similar observations were also made with other cow's milk proteins which are considered highly susceptible to proteolysis by digestive enzymes, i.e. lactalbumin (Maynard, 1997) and casein (Spuergin, 1996).

Several clinical studies have reported controversial results with hydrolyzed formulae, depending on the enzymes used and on the degree of hydrolysis. The incidences of reported adverse effects on babies fed partially or extensively hydrolyzed milk (either casein or whey) formulae range around 45-65% and 15% respectively (Oldaeus, 1991; Ragno, 1993; de Boissieu, 1997). When partially hydrolysed formulae are concerned allergic reactions may be

due to the presence of some residual native protein or of large fragments derived there from. When extensively hydrolyzed formulae are concerned, where no protein nor large fragments are still present, the allergic reaction may be triggered by short peptidic fragments comprising IgE-binding epitopes, such as those described above, that are released during the proteolysis.

In conclusion, depending on the the methods employed *in vitro* digestion has a heterogeneous effect on the allergenicity of BLG : it may leave it unaltered, reduce or enhance it. This suggests that the digesta still contain sufficient immunologically active structures (T-cell and B-cell epitopes) to potentially sensitize an individual or elicit an allergic reaction.

Conclusion

Stability to digestion has been considered by many as one of the properties shared by food allergens (Astwood, 1996). The use of digestion stability as a criterion for protein allergenicity assessment results from the general belief that for a protein to elicit an allergic response, it must survive the acid and proteolytic environment of the human gastrointestinal system to reach and be absorbed through the intestinal mucosa. Numerous food allergens have been shown to be stable to conditions simulating human gastrointestinal digestion. However, several recent investigations do not support the view that food allergens are necessarily more resistant to digestion than are non-allergenic proteins (Vieths, 1999; Yagami, 2000 ; Fu, 2002) or that their allergenicity is a consequence of their stability. The results presented here also show that allergenicity, at least IgE binding capacity, may remain after proteolysis whilst the intact protein has been extensively degraded and cannot be detected even at trace concentration. No protein does not imply no allergenicity and detection of intact known allergens in a food, even using improved analytical tools, is not a sufficient management measure to guarantee the likelihood of no or low allergenicity to allergic consumers.

Proteolysis as it occurs during the physiological process of digestion or when it is performed during *in vitro* digestibility tests as well as food processing in general may result either (or both) in destruction of conformational epitopes or/and in unmasking of existing epitopes and creation of new ones. As a consequence the allergenicity may be decreased, unchanged or even increased. The variability of the reported results highlights that the nature, structure and biological activity of peptides formed and released may be completely different depending on the conditions of the processing (e.g. hydrolysis) and particularly when these are produced

from a purified protein in a buffer solution (e.g. in a pepsin resistance test) to those produced in a complex biological medium, where interactions with other constituents may occur. Our data indicate that in assessing the impact of digestion on food protein allergenicity more than one parameter should be taken in to consideration. Thus the estimation of digestive stability is crucial, but the optimal form of the assay for assessing allergenic activity is also important

Resistance to pepsin digestion is still a relevant characteristic in the weight of evidence approach to assess the allergenicity of a novel protein (Codex Alimentarius, 2003 ; EFSA, 2004). However, our data support the idea that "resistance to digestion" may include two different types of reactions: a) persistence/non persistence of known allergenic molecules and b) appearance of new molecules from digestion which should be also recognized in the various biological assays. This study further demonstrates that a broad analytical and immunological approach is needed to cover a more complete picture of what allergenicity is like to meet the demanded standards for a complete and reliable assessment of allergenicity and to to minimize the risk of novel food components for consumers health.

Acknowlegments

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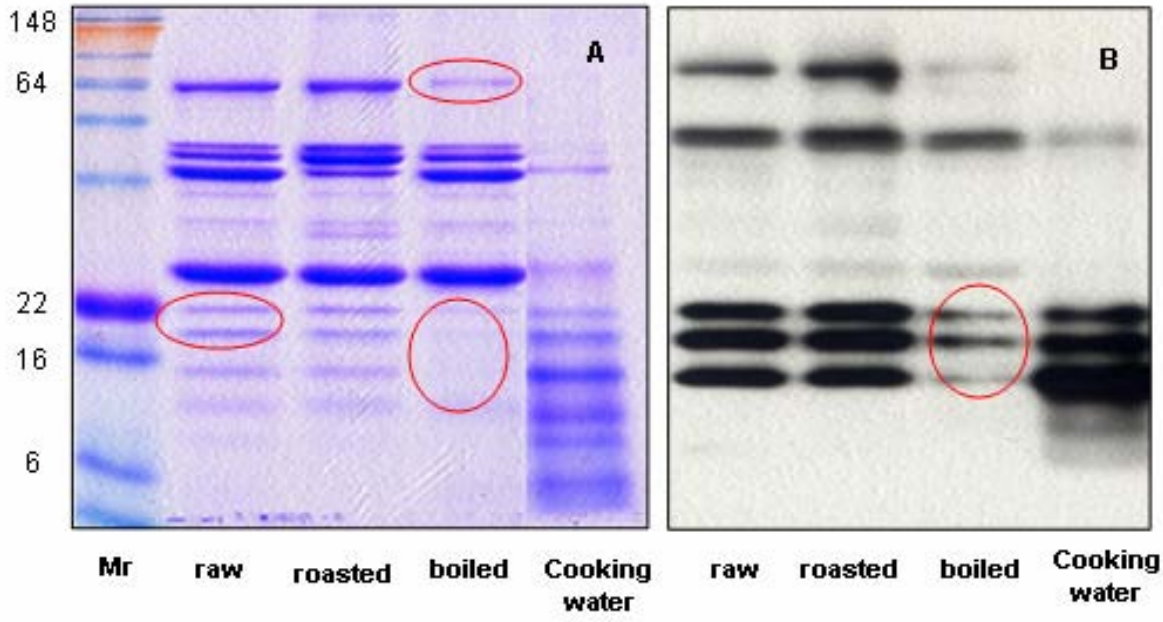
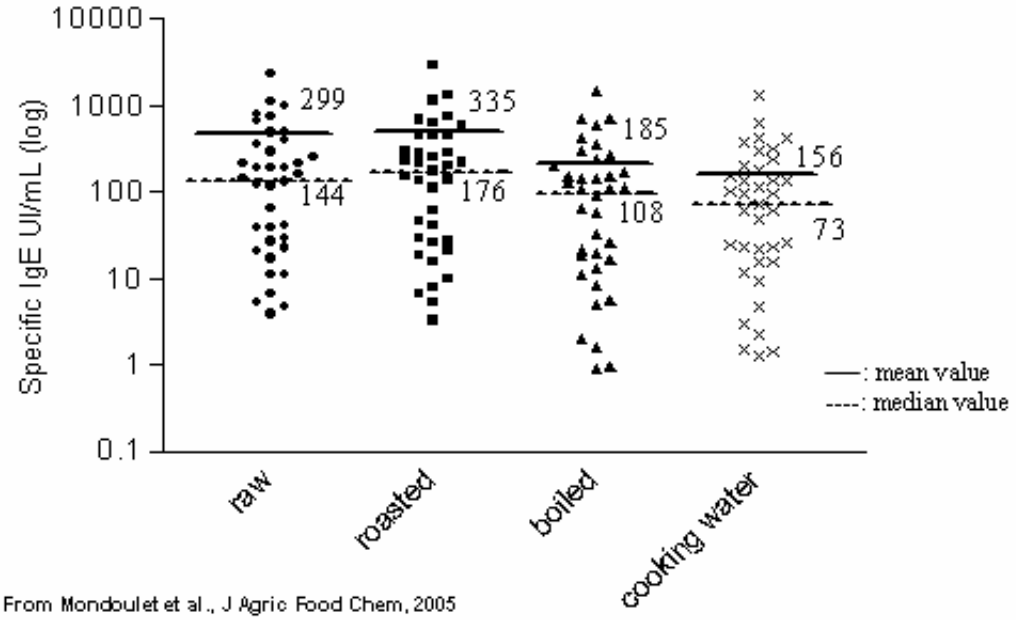
FIGURES

Fig. 1 : SDS-PAGE and western blot of whole peanut protein extracts after different heat treatments



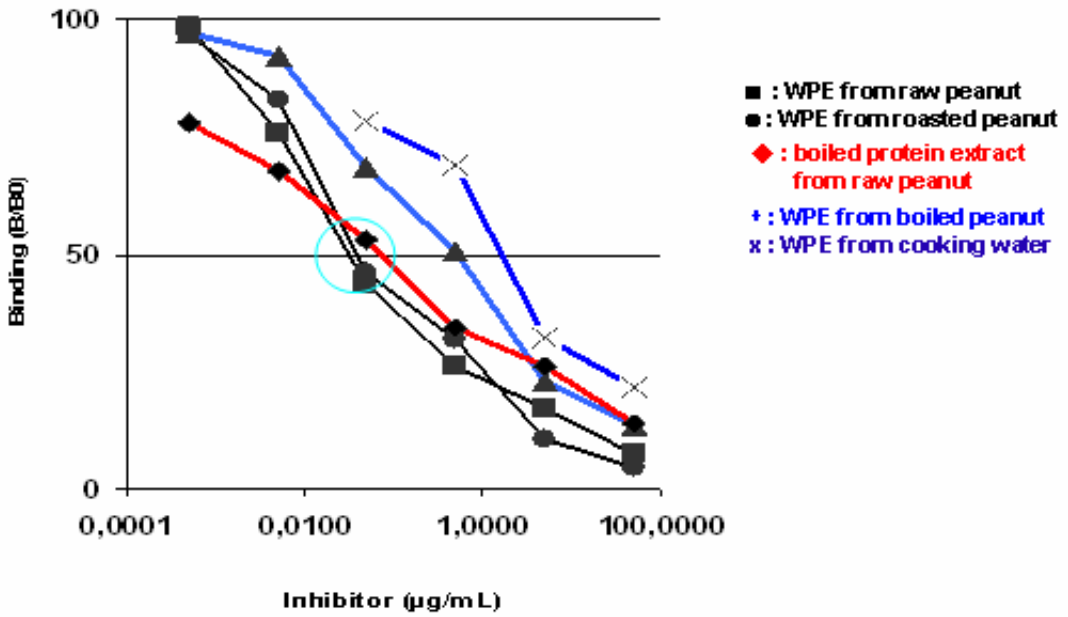
From Mondoulet et al., J Agric Food Chem, 2005

Fig; 2 : Specific IgE response to different whole peanut protein extracts



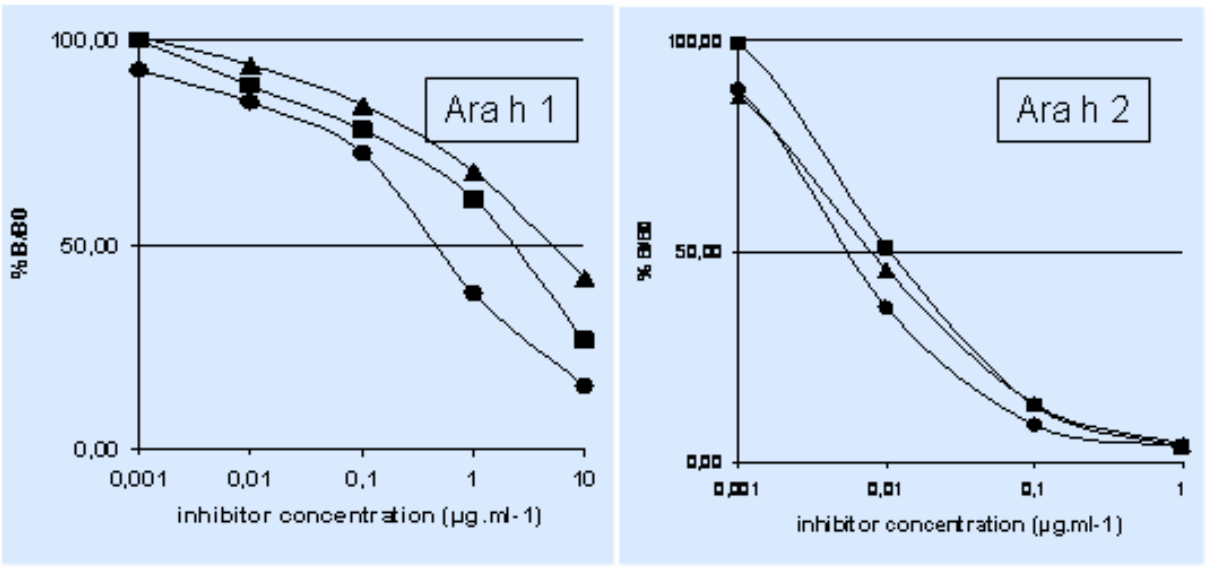
From Mondoulet et al., J Agric Food Chem, 2005

Fig; 3 : Effect of heat treatment on whole peanut protein extract (WPE) IgE binding



From Mondoulet et al., J Agric Food Chem, 2005

Fig; 4 : Effect of heat treatment on purified peanut protein IgE binding



From Mondoulet et al., J Agric Food Chem, 2005

Fig; 5 : Effects of heat treatment on milk proteins IgE binding

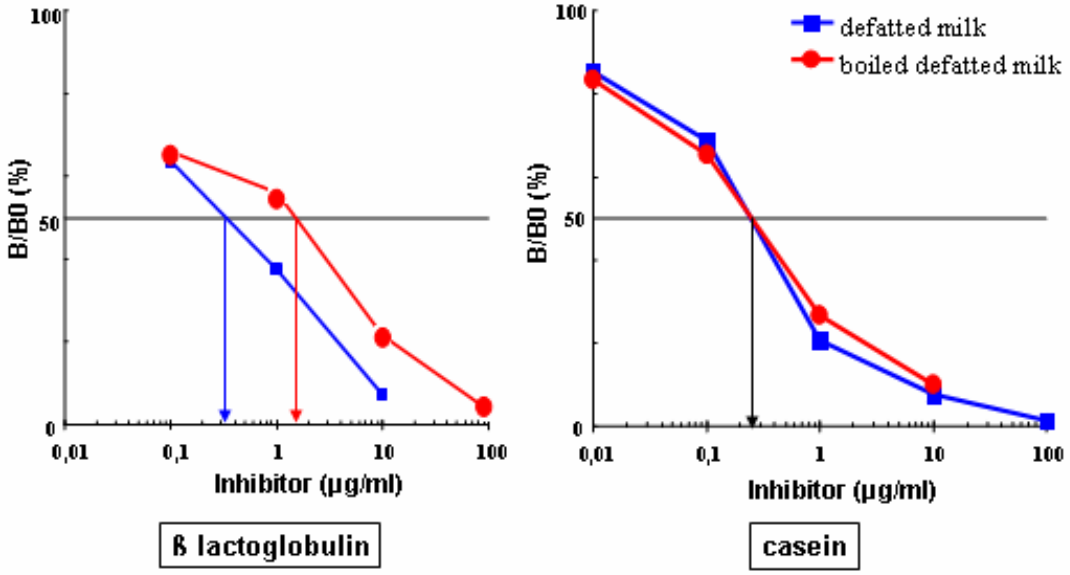


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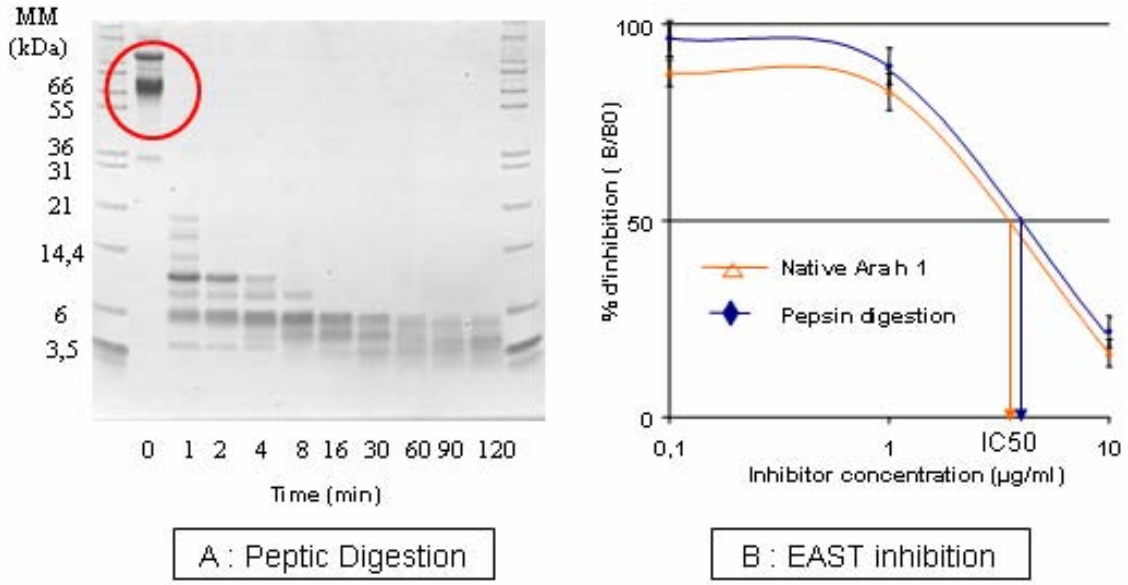
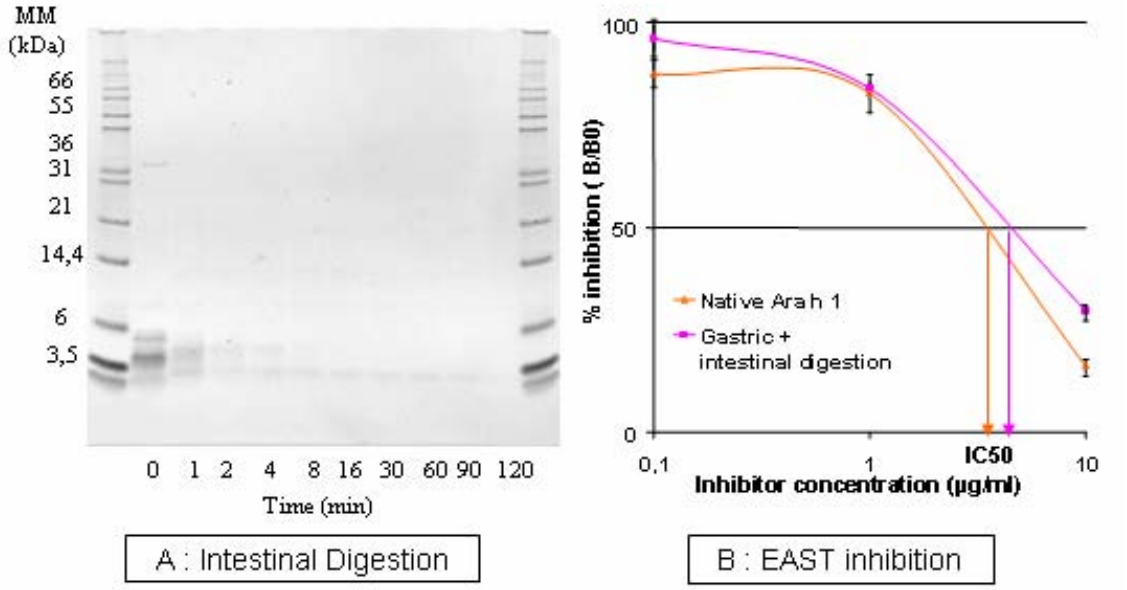
Fig. 6 : Effects of pepsin digestion on IgE binding capacity of Ara h1 and fragments



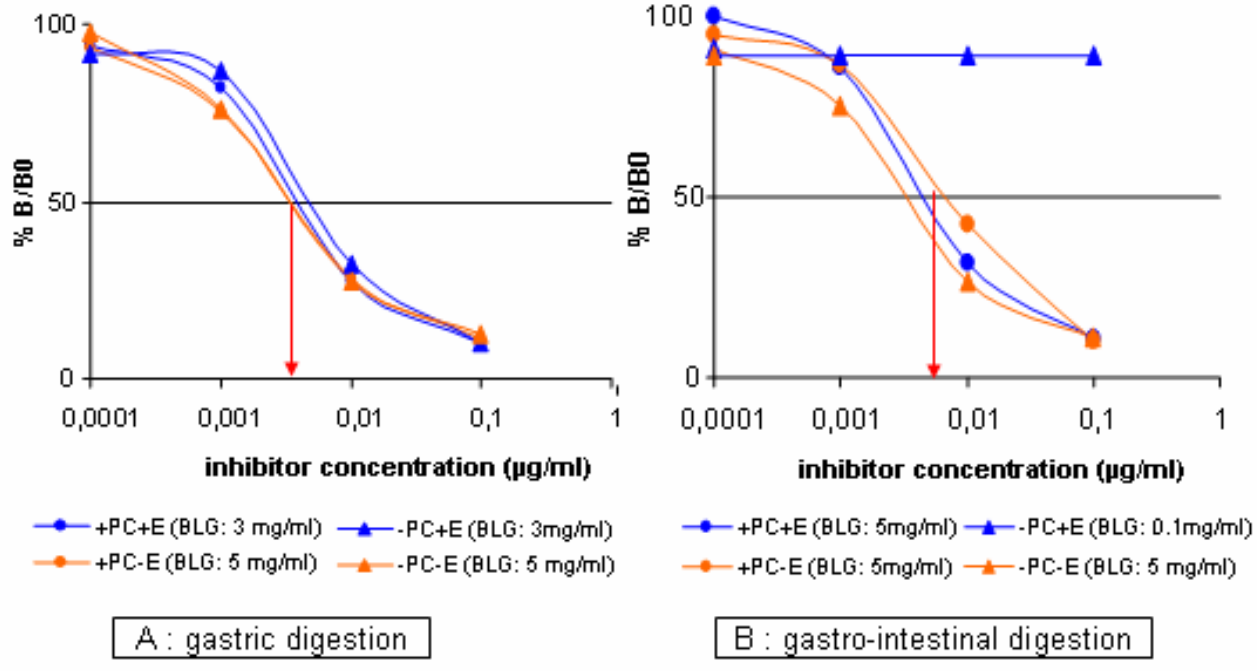
From Eiwegger et al. unpublished results from ALLERGEST project

Fig. 7 : Effects of gastro-intestinal digestion on IgE binding capacity of Ara h1 and fragments



From Eiwegger et al. unpublished results from ALLERGEST project

Fig. 8 : Effects of digestion on IgE binding of purified ß lactoglobulin



From Theodoropoulou et al., unpublished results. Allergest project