The Reactivity and Allergenic Potential of Hazelnut Peptides

Lavinia Florina CĂLINOIU, Dan Cristian VODNAR*, Carmen SOCACIU

Department of Food Science and Technology, University of Agricultural Science and Veterinary Medicine, Mănăștur Street, nr.3-5, 400372, Cluj-Napoca, Romania; *vodnar_dan@yahoo.co.uk

Abstract. The aim of this paper was to focus on proteins present in some food products, like hazelnuts and to investigate their allergenic potential. Several techniques were used to characterize these extracted proteins, with respect to their composition, degradability by digestive proteolytic enzymes and their reactivity with specific antibodies. It was important to analyse which proteins were present in the hazelnuts, to see if there were proteins present to trigger an allergic reaction and if the digestion enzymes trypsin and pepsin influence the presence of the (allergic) protein compounds.

Allergies to tree nuts and seeds can cause life-threatening and sometimes fatal reactions. To examine the properties of Hazelnut protein it was important to solubilize it by extraction. After extraction, it was investigated how hazelnut protein can be modified by proteases and what the effect was on the immune reaction.

The Bradford method is a fast and sensitive method to determine the concentration of soluble protein. When the Bradford reagent (Coomassie Brilliant Blue) binds to the protein, the colour changes from red to purple and the absorption maximum changes from 495 to 595 nm. The value obtained as the final concentration of proteins was 7.3495.

SDS-PAGE is a method to separate mixtures of proteins by electrophoresis. Protein molecules are negatively charged by binding of SDS molecules; subsequently they are separated in an electric field. Their differences in size (molecular weight) leads to separation. In this case the method is used to follow proteolytic degradation of hazelnut proteins (allergens) by intestinal proteases (trypsin, pepsin).

A different, more specific and sensitive method is immunoblotting (Western Blot) in which the SDS-PAGE separated proteins are transferred from the gel to a membrane and specific antibodies are used in a series of reactions to visualize specific allergens on this membrane. The remarked spots represented a positive identification of allergenic proteins. This means that peptide fragments of various size, produced during the digestion of a protein can still be immunological active. As it was shown there was still reactivity between proteins and specific antibodies.

The Dot Blot is a simple immunoblotting technique used to detected specific proteins in a mixture of different proteins and/or other molecules. No separation technique prior to the actual immuno-detection is necessary. Also, Dot Blot confirmed the presence of allergenic proteins made visible through the light spots on the membrane.

Keywords: Bradford method, SDS-PAGE, Western-Blot, Dot-Blot

INTRODUCTION

The immune system is a network of cells, tissues and organs that work together to defend the body against attacks by “foreign” invaders.

Long ago, physicians realized that people who had recovered from the plague would never get it again—they had acquired immunity. This is because some of the activated T and B cells become memory cells. The next time an individual meets up with the same antigen, the immune system is set to demolish it. Immunity can be strong or weak, short-lived or long-lasting, depending on the type of antigen, the amount of antigen, and the route by which it
enters the body. Peanut and tree nuts allergy can be particularly dangerous because allergic attacks can occur on the first known exposure to trace amounts of peanut due to inadvertent sensitization from the environment. There is some slight evidence that oral administration of peanuts can be protective in infants receiving high environmental exposure (Brandtzæg P., 2010).

In a very interesting study comparing peanut and tree nuts allergy between UK and Israeli Jews, the low incidence of peanut and tree nuts allergy in the latter may have been due to early consumption of peanuts in very young children in Israel (Asher MI et al., 2006).

Recent studies showed a prevalence of peanut and tree nut allergy of 1-2% in the US and Canadian population. In some studies the prevalence was found to be much higher, with approximately 10% of 8-year-old children in the UK being sensitised to peanut, of whom 2% have a clinical peanut allergy. In addition, the prevalence of peanut and tree nut allergy in children appears to be increasing, which contributes to a growing global concern, particularly given the severity of the allergic reactions (Sicherer SH, Munoz-Furlong A, Godbold JH, Sampson HA, 2010).

Food allergens are named by the first three letters of the taxonomic name of the genus of its botanical source, followed by the first letter of the species, and an Arabic number to indicate the chronology of allergen discovery and purification. As example, the first described allergen of peanut (Arachis hypogaea) is named, Ara h 1. Peanuts and tree nuts are known to cause the largest number of cases of severe anaphylaxis and deaths in the US. In general these allergies are triggered by the major proteins found in nuts and seeds which are resistant to processes such as cooking. Hazelnuts (Corylus avellana) contain about 15% protein and 60-65% lipids. Peanuts consist of approximately 45-50% oil, 25-30% protein, 8-12% carbohydrates, 5% water, 3% fibre and 2.5% ash. Cross-reactivity of peanut and tree nut allergens could contribute to the high incidence of tree nut sensitisation in peanut allergic individuals. Sera of peanut-allergic patients that strongly interact with Ara h 1 were shown to often react with the corresponding vicilin allergens (Cor a 11, Ana o 1, Jug r 2) from tree nuts (Aalberse RC, 2007).

Antigenicity, which is the ability of an epitope to react with an antibody, must be distinguished from its immunogenicity or ability to induce antibodies in a competent vertebrate host. To a clinical allergist, antigenicity reflects the capacity of an allergen to induce symptoms or a skin reaction, whereas to an immunologist, it reflects either a peculiar type of immunogenicity (ie, the capacity of a protein to induce IgE antibodies) or simply the capacity to bind IgE antibodies (Traidl-Hoffmann C, Jakob T, Behrendt H, 2009).

More knowledge on the influence of food processing, digestion and the food matrix on antigenicity of individual allergens is important in diagnostic procedures.

Food processing offers opportunities to alter the nature of epitopes of a protein, which is important for immune recognition by food component-specific antibodies. For example, three-dimensional epitope conformation may be modified as a result of protein denaturation treatments (e.g. various thermal processing treatments) leading to destruction or break up of epitopes and therefore leading to reduction in IgE binding capacity. However, denaturation treatments can also lead to generation of new epitopes, or to exposure of formerly hidden antigenic sites, which results in an increase in IgE binding. Acid or enzyme hydrolysis of an allergenic protein may help to delete critical amino acids of an epitope. Whether caused by protein denaturation or hydrolysis, loss of epitope and ensuing loss of IgE binding may help to reduce the bioactivity of an allergen (Mills EN, Sancho AI, Rigby NM, Jenkins JA, Mackie AR, 2009).

Due to the very acidic conditions in the stomach and the intense proteolysis occurring in the stomach and intestine, only small amounts of intact or immunologically
active proteins are taken up by the gut mucosa. This suggested that food allergens are, at least partially, resistant to gastro-duodenal digestion in order to be able to sensitize the mucosal immune system. Susceptibility to digestion has therefore been considered an important biomarker for food allergy, the idea being that proteins or at least peptides of a few kDa that survive digestion are more likely to be allergens. Whilst this idea has some appeal, the evidence is rather equivocal; showing that peptide fragments of various sizes, produced during the digestion of a protein can still be immunologically active. Furthermore, it is shown that some food allergens are rapidly and extensively degraded during digestion, whereas some other food proteins that are resistant to digestion are not allergenic (Adel-Patient K, Wal JM, 2008).

A pregastric absorption also occurs, i.e. in the oral cavity, which explains the occurrence of symptoms few minutes after ingestion of food allergens. Furthermore, even though the immune system is mostly exposed to digested proteins, it is, in addition, exposed to intact allergens to a lesser extent. It is assumed that a small fraction of intact dietary proteins are absorbed from the mature gut, thus leaving the option open that undigested proteins play a role in the allergic sensitisation process (Gardner MLG, 1988).

A review on intestinal barrier function also summarized evidence from studies demonstrating that intestinal barrier dysfunction leading to increased intestinal permeability and exposure to intact proteins which in turn might promote sensitization and enhance the severity of food induced allergic reactions. Additionally, it has been widely documented both in humans and in animal models, that an increase in gastric pH (e.g. caused by antacid therapies) impedes the gastric protein digestion and presumably facilitates the presentation of food peptides to intestinal T cells (Groschwitz KR, Hogan SP, 2009).

The aim of this paper was to characterize the extracted proteins, with respect to their composition, degradability by digestive proteolytic enzymes and their reactivity with specific antibodies.

MATERIALS AND METHODS

The experimental part has been carried out in Holland at Wageningen University, in the laboratory of food allergies. First, an experimental design was made which represented the research pathway (Fig. 1).
The first step was to take the hazelnuts and make the extraction in order to solubilize them. The proteins dissolved slowly in the buffer (Tris buffer pH 7.5) and the insoluble materials were being removed by centrifugation (12000 g for 20 min in a high speed centrifuge Beckmann). The supernatant represented the main key in all of the next processes because there were the proteins of interest.

The Bradford test was done to determine the concentration of the soluble protein. The first samples were used for a standard curve (using 2 mg BSA/ml stock solution 7 dilutions were prepared). With this standard curve was calculated the protein concentration by using the formula from the trend-line. The absorbance measured from the proteins represented the y-value through which was measured the x-value which represented the concentration of the protein in mg/mL. Therefore, the concentration of the soluble protein was known and the protein digestion could be started.

The HazNut solution (solution of hazel nut proteins, pH 2.3) was treated with pepsin, which mimics the protein digestion in the stomach, and trypsin which mimics the protein digestion in the small intestine in order to investigate the proteolytic degradation of extracted hazelnut protein and the effect of this on the immune-reaction with antibodies.

After the proteolytic degradation of hazelnut proteins by intestinal protease, the samples were subjected to the SDS-page method. The proteins are separated in an electric field (Biorad Power Pac 300; power supply to 200V constant). Their differences in size (molecular weight) leads to separation: small molecules run faster than large ones. This electrophoresis separation was required for the next experiment on the immune assay (Western Blot), experiment which needed a prior separation of the proteins. The Western blot is an immunoblotting technique used to detect specific proteins immobilized on a membrane. The detection of the protein of interest proceeded in three steps. First, the membrane (PVDF membrane) was incubated (with agitation for 30 min at 37°C) with the primary antibody (raised in mice against that specific protein), which binds only to the protein of interest. Next, a secondary antibody (anti-mouse IgG), which recognizes an epitope on the first antibody was incubated with the membrane. The second antibody was conjugated to a labeling agent such as the enzyme alkaline phosphatase. Finally this marker was then visualized by a colorimetric reaction (NBT/BCIP substrate solution) catalyzed by this enzyme which yielded a colored product that remained fixed to the membrane.

Another immunological technique used to test the reactivity of the extracted protein was the Dot Blot method. The difference from the Western Blot method is that no separation technique prior to the actual immune-detection was necessary. The sample containing the protein of interest was directly applied onto a protein-binding membrane (PVDF membrane).

RESULTS AND DISCUSSIONS

The final concentration of the extracted sample was determined with the Bradford technique. For this, a calibration curve of BSA solution had to be done. The absorbance measured for those two samples (UD, 5Xd) were in the range, the highest value on the absorbance scale being 1,8 (Tab. 1).

<table>
<thead>
<tr>
<th>Bradford Samples</th>
<th>Absorbance 595 nm</th>
<th>Concentration (x) obtained after applying the equation (y=0.6765x+0.5036)</th>
<th>Final protein concentration(x*5) mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein undiluted(UD)</td>
<td>1.213</td>
<td>1.4699</td>
<td>7.3495</td>
</tr>
<tr>
<td>Protein diluted(5x)</td>
<td>1.498</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tab. 1
By looking at the results from the Bradford (Tab. 1) it could be concluded that this procedure went well and the $R^2$ shows that the trend line was quite accurate (Fig. 2).

The final concentration was calculated with the equation on the chart (Fig. 2), where:

- $y = $ absorbance of 5xd sample
- $x = $ concentration of the 5xd sample (this value multiplied by 5 gives the final concentration of the proteins from the sample).

The value obtained as the final concentration of proteins was acceptable (Tab. 1.), even good and in comparison with the amount of proteins illustrated on the nuts package (14 mg/mL) it was more than half from it, which was explainable because the total amount of proteins contains both, soluble and insoluble proteins (in this case only the soluble ones were examined) and the extraction time was short due to the high number of experiments that had to be done (a longer extraction time leads to a better extraction).

The SDS-PAGE method was used to follow proteolytic degradation of hazelnut proteins by intestinal proteases. With the help of the PageRuler Prestained Protein Leader (Fig. 3) it was compared the SDS-page separated proteins membrane with the standard and concluded if the degradations were efficiently done (partially or totally) on the base of their size (small molecules run faster so they will be represented, more or less, on the bottom of the membrane, while the large molecules will occur on the superior part of the membrane).

For interpreting the results obtained after this experiment, the Western Blot membrane (Fig. 4) will be taken into consideration. How higher the line in the gel how higher
the molecular weight and how thicker the line how more proteins there are of that weight. The first thing that can be observed was that the Mwm Protein ladder (ladder 8) was quite clear. The obtained membrane was very well usable to see differences between the samples and to read the molecular weights. Sample 1, the undigested protein sample indicates that the extraction went well (lots of different proteins).

The Western Blot technique is qualitative, but has some important advantages like: allows positive identification of allergenic protein, if the band, stained in the immunoblot, correspond with a known allergen; allows detection of all proteins present, if they contain a linear epitope and if they are larger than 3000Da. Only at the spots where enzyme-antibody is bound a local enzyme reaction takes place leading to the conversion of a soluble substrate into an insoluble colored product. As a result, the spots containing the antigen will stain. This kind of spots can be remarked also on the analyzed membrane (Fig. 4). This means that peptide fragments of various size, produced during the digestion of a protein can still be immunological active. As it was shown there was still reactivity between proteins and specific antibodies. In the last two columns, due to the fact that there were loaded only pure samples of pepsin and trypsin (without proteins) no marks (spots) were present.

For analyzing the different samples and in particular how the digestion of proteins influenced the presence of proteins, it was necessary to compare the Western Blot membrane with the standard (Fig. 3), results which were presented in the table below (Tab. 2).

**Western Blot results indicating the weight of proteins found**

<table>
<thead>
<tr>
<th>Western-blot</th>
<th>10-&gt;25 kDa</th>
<th>26-&gt;35 kDa</th>
<th>40 kDa</th>
<th>55 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude hazelnut extract</td>
<td>15</td>
<td>35</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Pepsin 1 min.</td>
<td>14, 15, 20, 25</td>
<td>35</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Pepsin 10 min.</td>
<td>10, 11, 12, 13, 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pepsin 30 min.</td>
<td>10, 11, 12, 13, 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin 1 min.</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin 15 min</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin 30 min.</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pep + Tryp 15 min.</td>
<td>10 (extremely little)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pep + Tryp 30 min.</td>
<td>10 (extremely little)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
When analyzing the exposure to trypsin, practically there was no change in the proteins content/composition the longer the sample was in contact with the trypsin. Since trypsin cleaves to proteins and hydrolyses them in smaller peptides, was expected a reduction of the large molecular weight peptides, which it could not been seen. The hydrolyzation was already done by the time of the first measure, since in comparison to the first sample (undigested protein sample), the large proteins were already gone. Was presumed that it was correct, thus it was believed that the reason no change was seen, was the breakdown of the peptide which occured very quickly (before the first measurement). Some 25 kDa proteins were found in all 3 trypsin rows, meaning there were some proteins present that are highly resistant to trypsin. Prolonged incubation had little to no influence on the digestion of proteins by trypsin.

For the pepsin, there was a clear decrease in high molecular weight proteins. This means that the pepsin needed some time to cause full hydrolysis of the proteins into small peptides. Eventually only peptides of around 10 kDa were left. This is in line with literature data (Tatiana Cucu, Céline Platteeau, Isabel Taverniers, Bart Devreese, Marc De Loose, Bruno De Meulenaer, 2013) where the hydrolysis took place fast for the hazelnut proteins as manifested by the losses of intensity of the main protein bands with formation of peptides with masses between 10 and 15 kDa and the degradation of the main protein bands took place as soon as after 30 min of hydrolysis.

In the case of exposure to both, pepsin and trypsin, no (measurable) peptides were present anymore. This means that a combination of both enzymes would speed up the hydrolyzation immensely and would result in peptides only smaller than 10 kDa.

For the Dot-Blot, 2 membranes were used: + primary antibody (Fig. 5) and - primary antibody which is a control without primary antibody (Fig. 6). By the results obtained, the membrane was not very clear (Fig. 5), but it confirmed the presence of allergenic proteins made visible through the light spots on the membrane.

CONCLUSION

As a conclusion, these several techniques were used to determine the concentration of the proteins from hazelnut in order to subject them to a proteolytic digestion for finding out their degradability (partially or totally) so, in the end, to investigate if they still could lead to allergies by testing their reactivity with specific antibodies.

The value obtained as the final concentration of proteins was acceptable and in comparison with the amount of proteins illustrated on the nuts package (14 mg/mL) it was more than half from it.

Susceptibility to digestion has therefore been considered an important biomarker for food allergy, the idea being that proteins or at least peptides of a few kDa that survive digestion are more likely to be allergens.
Peptide fragments of various sizes, produced during the digestion of a protein can still be immunological active.

An increase in gastric pH (e.g. caused by antacid therapies) impedes the gastric protein digestion and presumably facilitates the presentation of food peptides to intestinal T cells. Changes in protein structure by processing methods might enhance or decrease IgE binding, but the changes by processing might also enhance or hinder the digestibility of a particular protein.

Due to the very acidic conditions in the stomach and the intense proteolysis occurring in the stomach and intestine, only small amounts of intact or immunologically active proteins are taken up by the gut mucosa.

REFERENCES