

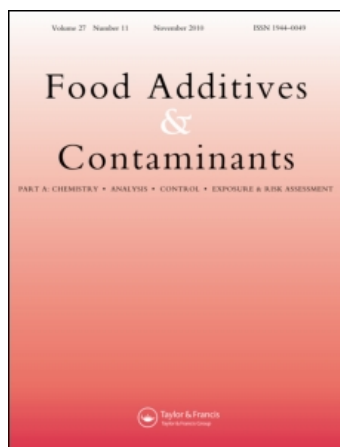
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C. Platteau^{ab}; T. Cucu^{ac}; B. De Meulenaer^a; B. Devreese^c; M. De Loose^{bd}; I. Taverniers^b

^a Department of Food Safety and Food Quality, Research Group Food Chemistry and Human Nutrition, Ghent University, Coupure Links 653, B-9000, Belgium ^b Flemish Government, Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium ^c Department of Biochemistry and Microbiology, Laboratory for Protein Biochemistry and Biomolecular Engineering, Ghent University, Ghent, Belgium ^d Department of Molecular Genetics, Ghent University, Ghent, Belgium

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Effect of protein glycation in the presence or absence of wheat proteins on detection of soybean proteins by commercial ELISA

C. Platteau^{ab}, T. Cucu^{ac}, B. De Meulenaer^{a*}, B. Devreese^c, M. De Loose^{bd} and I. Taverniers^b

^aDepartment of Food Safety and Food Quality, Research Group Food Chemistry and Human Nutrition, Ghent University, Coupure Links 653, B-9000, Belgium; ^bFlemish Government, Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium; ^cDepartment of Biochemistry and Microbiology, Laboratory for Protein Biochemistry and Biomolecular Engineering, Ghent University, Ghent, Belgium; ^dDepartment of Molecular Genetics, Ghent University, Ghent, Belgium

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Soybean (*Glycine max*) is the world's primary provider of protein and oil and is widely used in foodstuffs. However, the use of soybean in foodstuffs might pose a serious threat to allergic consumers since some proteins can cause allergic reactions. To date mostly ELISA methods are used for testing contamination of foodstuffs with soybean. In view of the complexity regarding allergen detection in foodstuffs and appropriate food product labelling, the aim of this study was to investigate the impact of the Maillard reaction on the detectability of soybean proteins using commercial ELISA kits. Accumulation of protein-bound carbonyls, modification of reactive lysine residues and severe aggregation as a result of incubation with glucose, in the presence or absence of soluble wheat proteins, were recorded. Moreover, detection of soybean proteins by means of three commercial ELISA kits was strongly altered and was highly dependent on the type of kit used.

Keywords: soybean allergy; Maillard reaction; ELISA; protein bound carbonyls; protein aggregation; reactive lysine

Introduction

Food allergy is an abnormal immunological response due to sensitisation to a food component. With its increasing incidence over the last few years, it represents an important health problem, especially in industrialised countries where it has been estimated to affect about 2% of the adult population and up to 8% of children (Monaci et al. 2006). Together with hen's egg, cow's milk, fish, crustacea, peanut, tree nuts and wheat, soybeans belong to the 'big eight', being responsible for 90% of all allergic reactions (Food and Agricultural Organization (FAO) 1995). It also belongs to the list of 14 allergenic products that have to be declared on the label of a food product when present as an ingredient according to Directive 2007/68/EU (European Union 2007).

Soybeans (*Glycine max*) contain about 35% of proteins and at least 21 proteins have been shown to be IgE reactive. Soybean allergy can occur as a result of primary sensitisation, but can also be related to birch pollen allergy through structural similarities between the birch pollen allergen *Bet v 1* and the corresponding protein *Gly m 4* in soybeans (secondary allergy) (Mittag et al. 2004; Ballmer-Weber and Vieths 2008).

The symptoms associated with soybean allergy are mostly mild, such as the Oral Allergy Syndrome (OAS); however, severe reactions have also been described (Foucard and Yman 1999; Kleine-Tebbe et al. 2002; Mittag et al. 2004). The incidence of soybean allergy has increased during the last years probably because more products are being produced with soybean (Wilson et al. 2005). Soybean is used as an ingredient in a broad range of food products, for both its texturising and nutritional benefits. Due to its diverse functional properties, such as water and fat absorption, emulsification, foaming and gelation, soybean proteins are often included during food processing steps. However, extensive use of soybean proteins in foods might pose a serious threat to the allergic consumers due to unintentional contamination that might take place during food processing. This can affect the safety of food products and lead to the presence of hidden allergens, therefore making avoidance diets increasingly challenging.

Various approaches have been applied to reduce the allergenicity of soybean proteins. Enzymatic hydrolysis through fermentation is often applied to produce hypoallergenic formulas, but is strongly

*Corresponding author. Email: Bruno.DeMeulenaer@UGent.be

dependent on the extent of the hydrolysis degree (Yamanishi et al. 1995; Tsuji et al. 1997; Frias et al. 2008). Reduction of soybean allergenicity has also been achieved by allergen conjugation with polysaccharides (Babiker et al. 1998; Arita et al. 2001). Similarly, the impact of protein modifications occurring as a result of the multitude of chemical reactions occurring during food processing has also been studied (Burks et al. 1991; Codina et al. 1998; Muller et al. 2000). In contrast to these studies focusing on the impact of soy protein modification on the antigenicity, only limited studies are available with respect to the impact of these changes. In addition, the impact of these changes on the detectability using immunochemically methods, which are typically used by the industry and enforcement agencies (Tsuji et al. 1995; Bando et al. 1998), has rarely been studied in a systematic manner for soybean protein. The above-mentioned methods are based on antibody–antigen interactions. Therefore, immunoassays are subjected to complications due to matrix effects, which may result in erroneous results. Moreover, the interaction of food allergens with other food components, which are expected to occur during thermal processing, may lead to changes in the allergen structure and might hinder their detection by immunological methods. Therefore, it is important to gain knowledge on the influence of food processing on the tertiary structure of food allergens and the changes occurring at the amino acid level as a result of chemical reactions taking place during processing. Moreover, information on the further impact of these changes on the allergenic potential and the detectability of the allergens is invaluable. Van de Lagemaat et al. (2007) previously reported reductions of the antigenicity of soybean proteins after *in vitro* glycation using an in-house-developed ELISA assay. In this study the impact of glycation in the presence or absence of other proteins on the detectability of soybean proteins by commercial kits was investigated. A similar study on hazelnut proteins already showed that the detection was drastically affected as a result of protein glycation (Cucu et al. 2010).

Materials and methods

Reagents and standards

Soluble wheat proteins ‘Meripro 711’ were kindly provided by Syral Belgium N.V. (Aalst, Belgium). Chemicals and standards of analytical grade were obtained from Sigma-Aldrich (Bornem, Belgium), VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium). Solvents were of analytical grade from Chem-Lab (Zedelgem, Belgium), except for hexane, which was of technical grade. Gel and standards for SDS-PAGE were from Bio-Rad (Nazareth, Belgium), while the Krypton protein stain was provided by

Thermo Scientific (Aalst, Belgium). The commercial ELISA kits used were Veratox® Soy Allergen (Neogen Corporation, Lansing, Michigan, USA), BioKits Soya Allergen Assay (Neogen Corporation, Lansing, Michigan, USA) and Soy Residue (ELISA Systems, Windsor, Queensland, Australia).

Soybean protein extraction

Soybeans were provided by Alpro (Wevelgem, Belgium) and Cargill (Mechelen, Belgium). A mixture was made by taking equal amounts of each kind. Soybeans were frozen with liquid nitrogen and ground in a two-step process, first with a blender (Moulinex, France) and then with an Ultra Turrax t25 (IKA, Wilmington, DE, USA).

Soybean protein extracts were obtained according to the protocol described by Yeung and Collins (1996) with slight modifications. Briefly, 100 g of ground beans were defatted with four times 1 L technical hexane. After an overnight evaporation of the solvent at ambient temperature, 10 g defatted powder were mixed with 100 ml of extraction buffer and mixed at 4°C over night. Protein extraction buffer consisted of a 1 M NaCl, 1.47 mM KH₂PO₄, 2.7 mM KCl and 10 mM Na₂HPO₄. Sodium azide 0.2 g l⁻¹ was added to the extraction buffer to avoid microbial growth. Further, a mixture of protease inhibitors (2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium diethyldithiocarbamate (DIECA), 0.5 mM benzamidine hydrochloride (BAHC) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) was added to the extraction buffer in order to increase the stability of the protein isolates during extraction and storage (Bjorksten et al. 1980). The protein mixtures were further centrifuged for 1 h at 4°C and 20,000 g to separate the lees. Subsequently, the clear supernatants were dialysed (Spectra/Por 3 membrane tubing, MWCO 3.5 kDa) using the same extraction buffer containing only 0.137 M NaCl, for 48 h at 4°C and then filtered through a 0.45 µm membrane filter (Puradisc FP 30, Novolab, Belgium). Finally, the protein solutions were lyophilised and stored at -20°C until further use. This extract will be considered below as the reference extract.

Maillard reaction in the absence of soluble wheat proteins

Before treatment, soybean protein extracts were dialysed against the 0.1 M phosphate buffer pH 7.4 to remove the nitrogen containing additives (Spectra/Por 3 membrane tubing, MWCO 3.5 kDa). The reaction systems were prepared in the same phosphate buffer pH 7.4 by making a solution containing 1% (w/v) soybean proteins and 6% (w/v) glucose. The reaction systems in closed falcon tubes were incubated in a water bath at 70°C for up to 48 h. A reaction

system without glucose was used as the control. At different time periods samples were retrieved from the water bath and placed in an ice bath to stop the reaction. Further, the modified protein solutions were immediately used for the determination of reactive lysine residues, protein-bound carbonyls and free amino groups. Aliquots for SDS-PAGE were frozen at -20°C until further use.

Maillard reaction in the presence of soluble wheat proteins

Two types of model systems were considered. One model system contained 0.25% (w/v) of soybean proteins with 0.75% (w/v) of wheat proteins and 6% (w/v) of glucose in the same 0.1 M phosphate buffer pH 7.4. Another model system contained 0.5% (w/v) soybean proteins with 0.5% (w/v) wheat proteins and 6% (w/v) glucose. Respective model systems without glucose were prepared as control. Further these systems were treated similarly as the model systems containing no soluble wheat proteins.

Assessment of the Maillard reaction

Determination of protein-bound carbonyls

Protein-bound carbonyls were measured after converting them to the corresponding 2,4-dinitrophenylhydrazones. Briefly, 300 μl protein solution were incubated with 400 μl 2,4-dinitrophenylhydrazine (DNPH) 10 mM in 2 M HCl. Further, the proteins were precipitated with 10% trichloroacetic acid (final concentration) and thoroughly washed with 1 ml ethanol/ethyl acetate (1:1) after derivatisation; hence, any unreacted DNPH or DNPH bound to any other components than protein was removed. Finally the pellet was redissolved in 8 M urea and absorbance was measured at 370 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer (Nazareth, Belgium). The carbonyl content was calculated using a molar absorption coefficient of $22,000\text{ M}^{-1}\text{ cm}^{-1}$ (Levine et al. 1994).

Loss of reactive lysine residues

The loss of reactive lysine residues was monitored using derivatisation with *ortho*-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol, which yields a fluorescent product with a maximum excitation wavelength of 340 nm and emission of 450 nm using a Spectramax Gemini XPS fluorimeter from Molecular Devices (Brussels, Belgium) (Ferrer et al. 2003). A standard of β -casein was used to prepare a calibration curve and samples were pre-treated and analysed using a fluorimeter as previously described (Morales et al. 1996).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein cross-linking or fragmentation was analysed by SDS-PAGE under reducing conditions. A 4% acrylamide stacking gel and a 10% acrylamide resolving gel were used for protein separation. An aliquot of 10 μl sample containing 6 μg of protein was added to 10 μl Laemmli sample buffer containing β -mercaptoethanol and heated at 95°C for 5 min. The samples were further loaded on the gel along with the molecular weight marker. Precision plus protein unstained standard was used as a reference to determine the molecular weight of proteins of interest. Krypton fluorescence staining was used for bands visualisation. The gels were further read using the Molecular Imager PhorosFX Plus from Bio Rad (Nazareth, Belgium) where the molecular weights of the proteins were recorded.

Protein determination

Protein determination was performed by determining the nitrogen content according to the Kjeldahl procedure (AOAC International 1981). A multiplication factor of 5.71 was used to convert nitrogen content to soybean protein content, and a factor of 6.25 to convert to soybean protein content for the mixtures of soybean and wheat protein. The non-protein nitrogen (NPN) fraction was determined in the supernatant after a previous protein precipitation with 15% TCA (final concentration).

ELISA assays

Samples of the above-described model systems were analysed using different commercial ELISA kits: Veratox[®] Soy Allergen, BioKits Soya Allergen and Soy Residue. These samples include the glucose-free model system containing only soybean proteins (system 1), the model system containing soybean proteins with glucose (system 2), the two model systems containing different ratio's of soybean proteins and soluble wheat proteins (0.5% soybean proteins +0.5% soluble wheat proteins (system 3) and 0.25% soybean proteins +0.75% soluble wheat proteins (system 4), respectively) and the two model systems 3 and 4 containing different ratio's of soybean proteins and soluble wheat proteins, in the addition of 6% glucose ((system 5) respectively (system 6)). Of the different model systems, samples retrieved after 0-, 24- and 48-h incubation at 70°C were analysed. The untreated sample (=0 h of thermal treatment) of the glucose-free model system (1) containing only soybean proteins is referred to as the reference sample. For each sample of the different model systems a dilution series was made (0–50–100–500–1500–1500 ng ml⁻¹ soybean

protein) in the extraction buffer/dilution solution of the respective kit. For the BioKits Soya Allergen Assay additional dilutions had to be analysed. The actual concentrations are the concentrations of soybean proteins based on the absolute protein determination using the Kjeldahl procedure and do not include the wheat proteins that were added in some of the model systems. The dilution series were analysed *in duplo* in a similar manner as sample extracts according to the respective manufacturers' manuals. Absorbance measurements were made in a microtiter plate reader (Multiskan MCC/340, Titertek, Huntsville, Alabama). Each kit was calibrated using the respective standard protein solutions included in each kit. The absorbance values were fitted to the protein concentration of the standard solutions, according to a four-parameter-logistic dose-response curve corresponding to Equation (1). This equation presents a standard way for the expression of immunoassay data:

$$\text{Absorbance} = \left[\frac{(b - a)}{\left(1 + \frac{\text{conc}}{c}\right)^d} \right] + a \quad (1)$$

where a is the maximum dose (protein concentration); b is the minimum concentration; c is the E_{50} -value (concentration at half-maximal saturation); d is the slope of the curve; and conc is the concentration of the standard or sample. Only the data obtained within the range of the respective standard curve were considered in the results. In case of the Veratox[®] Soy Allergen kit, the kit's procedure prescribes to fit the data to log-logit function, which is a linearisation of the four-parameter logistic function. For the Soy Residue and the BioKits Soya Allergen Assay no suggestion is made as to which function to use to fit the data. Data above or below the calibration range are indicated by an asterisk (*) or a 'zero', respectively, in the charts.

Results

Progress of the Maillard reaction

An increase in protein-bound carbonyl amounts as a result of the incubation in the presence of glucose was recorded. A five-fold increase in protein-bound carbonyls was recorded after 24-h incubation of 1% soybean with glucose while after 48-h incubation a ten-fold increase was observed (Figure 1). For the model system containing no glucose a two times increase was observed. Similarly, an increase in protein-bound carbonyls was observed when soluble wheat proteins were added to the model systems. After 48-h incubation with glucose a seven-fold increase in protein-bound carbonyls was observed in the case of the model system containing 0.5% soybean proteins. For the

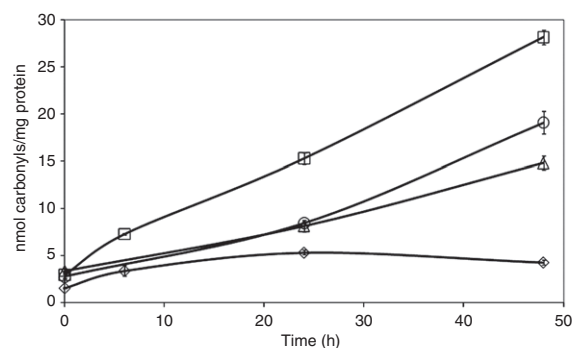


Figure 1. Formation of protein-bound carbonyls in soybean protein in the presence or absence of soluble wheat proteins due to incubation with glucose. Data points represent means $\pm$ SD of three independent determinations. □, 1% soybean proteins with 6% glucose; ○, 0.5% soybean with 0.5% soluble wheat proteins and 6% glucose; △, 0.25% soybean and 0.75% soluble wheat proteins and 6% glucose; and ◇, 1% soybean proteins.

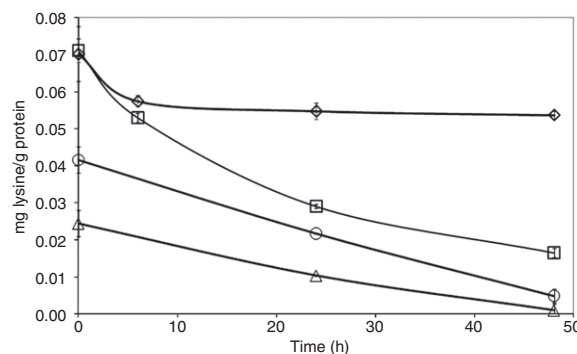


Figure 2. Loss of available lysine due to the incubation of soybean proteins in the presence or absence of soluble wheat proteins with glucose. Data points represent means $\pm$ SD of four independent determinations. □, 1% soybean proteins with 6% glucose; ○, 0.5% soybean with 0.5% soluble wheat proteins and 6% glucose; △, 0.25% soybean and 0.75% soluble wheat proteins and 6% glucose; and ◇, 1% soybean proteins.

model system containing 0.25% soybean proteins a four-fold increase was observed.

Further, changes in the reactive lysine residues were recorded. About an 80% decrease of reactive lysine residues was recorded upon incubation of 1% soybean proteins with glucose (Figure 2). Interestingly, a 23% decrease in reactive lysine residues after 6-h incubation in the absence of glucose was also recorded. A further decrease in reactive lysine residues was, however, not recorded, while in the presence of glucose a steady decrease was obtained throughout the whole incubation experiment. Further, about 90% of the reactive lysine residues were modified in the model system containing 0.5% soybean proteins after 48-h incubation with glucose while for the model system containing 0.25% soybean proteins almost all the reactive lysine residues were modified.

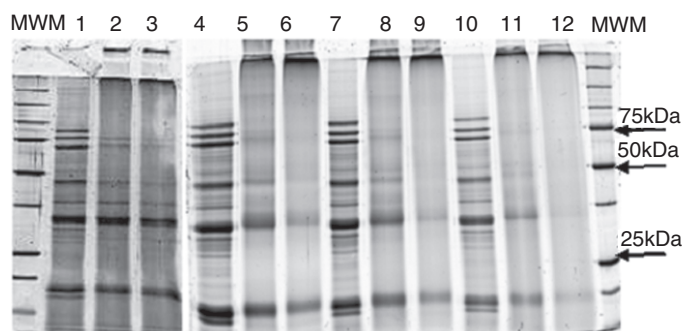


Figure 3. SDS-PAGE pattern of the soybean proteins incubated in the presence or absence of wheat proteins with glucose. Lanes 1–3, 1% soybean proteins non-treated, 24 h and 48 h incubated respectively; lanes 4–6, 1% soybean proteins and 6% glucose non-treated, 24 h and 48 h incubated respectively; lanes 7–9, 0.5% soybean proteins with 0.5% wheat proteins and 6% glucose non-treated, 24 h and 48 h incubated respectively; lanes 10–12, 0.25% soybean proteins with 0.75% wheat proteins and 6% glucose non-treated, 24 h and 48 h incubated respectively; and MWM, molecular weight marker.

Figure 3 shows the SDS-PAGE pattern of the native and glycosylated soybean proteins. Changes in the SDS-PAGE pattern of the soybean proteins incubated in the absence of glucose were observed (lanes 1–3). The intensity of the bands representing the α -, α' - and β - chains of the *Gly m 5* allergen with the molecular weight of 70, 74 and 50 kDa, respectively, decreased markedly with the incubation time (International Union of Immunological Societies 2010; Swiss Institute of Bioinformatics 2010). However, the acid and basic subunits of the *Gly m 6* allergen (32 and 19 kDa, respectively) remained stable upon the whole incubation time. More severe changes were observed, however, in the SDS-PAGE pattern of the soybean proteins incubated with glucose either in the presence or absence of soluble wheat proteins. Most of the allergenic proteins have been affected as manifested by the loss of intensity of their respective bands. Similarly as with the incubation in the absence of glucose, the α -, α' - and β -chains of the *Gly m 5* allergen were affected the most with the acid and basic subunits of the *Gly m 6* allergen being the most resistant. The respective bands of the *Gly m 6* allergen were still visible after 24-h incubation in the presence of glucose, although with slightly faded intensity. All the other proteins were highly modified as evidenced by the smearing towards the top of the gel which progressed with incubation time. After 48 h of incubation with glucose only the bands corresponding to the acid and basic subunits of the *Gly m 6* allergen were still slightly visible. Aggregates with masses of over 300 kDa were also formed that hindered their migration into the resolving gel.

Detection with commercial ELISA kits

The soybean protein concentration of the standards provided by each individual kit is expressed in parts

per million (ppm), which equals $\text{ng analyte mg}^{-1}$ of food product. When testing the commercial kits, the objective was to compare the actual soybean protein concentration of the analysed samples with the measured concentration, derived from the calibration curve obtained with the kit's standards. To this end, the concentration of the kits standards was converted to the same dimension of that from the model system samples. This was done according to Equation (2), considering the weight of the sample (mg) extracted by a particular volume of extraction buffer (ml):

$$\frac{\text{ng analyte}}{\text{mg food}} (\text{ppm}) \frac{\text{weight sample (mg)}}{\text{volume buffer (ml)}} = \frac{\text{ng analyte}}{\text{ml solution}} \quad (2)$$

For the evaluation of the kits' performance, dilution series of the soy protein solutions obtained from the six different model systems were applied in the appropriate concentration range of each kit. The obtained absorbance values were converted to a measured soy protein concentration, which was compared with the actual protein concentration present in each diluted solution (Figure 4).

BioKits Soya Allergen

With the standard solutions included in the BioKits Soya Allergen assay, a calibration curve was constructed according to the four-parameter logistic model, ranging from zero to 200 ng ml^{-1} soybean protein. The soybean protein concentration in the reference samples was overestimated on average by 290% in a concentration-dependent manner (Figure 4a). Therefore, at the highest concentrations absorbance values exceeding those obtained in the calibration curve were observed. Upon heating at 70°C without glucose for 24 h the signal decreased by 14% (data not shown) and an additional 8% when the treatment was extended

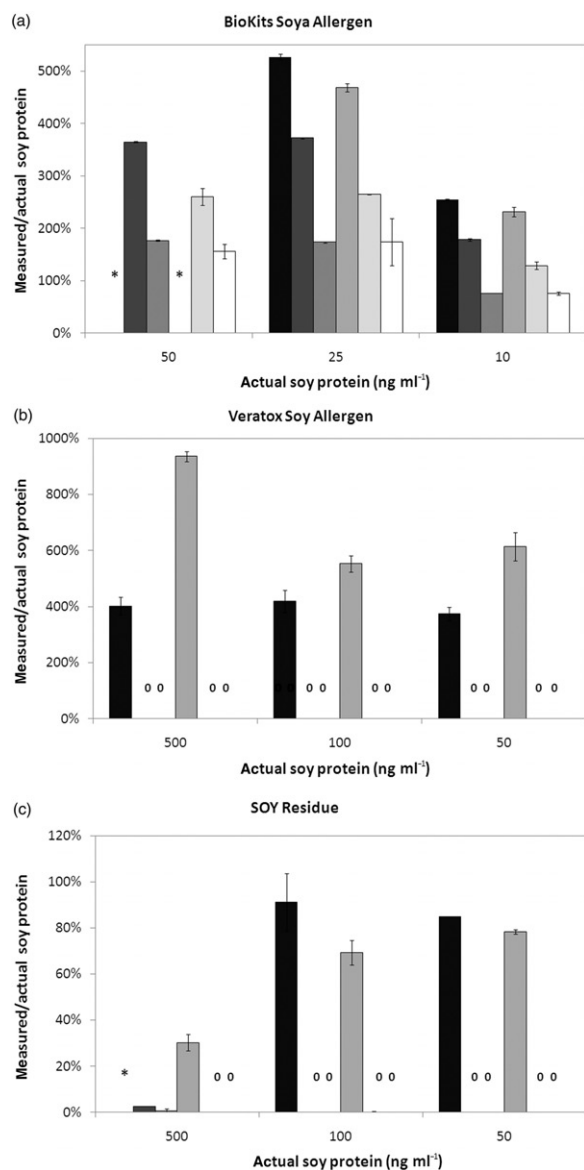


Figure 4. Ratio of measured over actual soybean protein concentration in different commercial ELISA kits of a dilution series of a reference soybean protein solution and after heating in the presence or absence of glucose and soluble wheat proteins. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range). ■, reference sample, ■, reference heated for 48 h, ■, reference heated for 48 h in the presence of glucose, ■, 0.25% soybean proteins and 0.75% soluble wheat proteins, ■, 0.25% soybean proteins and 0.75% soluble wheat proteins heated for 48 h, □, 0.25% soybean proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose.

up to 48 h. When soluble wheat proteins were added to the model system the average overestimation of the concentration of the samples was diminished to 250%, irrespective of the soy/wheat protein ratio. However, at the lowest soybean/wheat protein ratio a sharper decline in the measured soybean protein concentration was observed upon heating; after 48 h the signal

dropped on average by 38% (0.75% soluble wheat proteins) compared with an average of 27% (0.5% soluble wheat proteins; data not shown). The addition of glucose resulted in a further decrease of the measured concentration compared with the samples treated without glucose; the signal declined by 60% after 48-h heat treatment.

Veratox[®] Soy Allergen

A calibration curve was established with the standard solutions provided in the Veratox[®] Soy Allergen kit. The standard consists of Soy Protein Isolate and covers a range from zero to 4000 ng ml⁻¹ after conversion of the concentration expressed in ppm (ng mg⁻¹), according to Equation (2). As can be observed from Figure 4(b), the actual soybean protein concentration present in the reference solution and its dilutions was highly overestimated on average by 300%. Therefore, the obtained absorbance values for the reference samples with 1500 and 1000 ng ml⁻¹ soybean protein were beyond the range of the calibration curve and were not included in Figure 4(b). Upon the presence of soluble wheat proteins in the model system, the overestimation was even increased up to on average 400% (0.5% soybean protein + 0.5% soluble wheat proteins; data not shown) or on average 600% (0.25% soybean proteins + 0.75% soluble wheat proteins). However, after heating the reference sample for 24 h in the absence of glucose, the signal disappeared completely. In the presence of soluble wheat proteins only 1% (0.5% soluble wheat proteins) or 8% (0.75% soluble wheat proteins) of the soybean proteins were detected after 24-h heating (data not shown) and extension to 48 h led to a complete loss of signal. Heating the proteins in the presence of glucose confirmed the lack of robustness of the kits.

Soy Residue

In the Soy Residue assay the soybean trypsin inhibitor is used as the analytical target. The kit includes standard solutions containing 5, 2.5, 1 and 0 ppm soybean protein corresponding to 500, 250, 100 and 0 ng ml⁻¹ soybean protein after conversion which were used to construct a four-parameter logistic calibration curve. The 5 and 2.5 ppm standards returned equal absorbance values, amounting to 2.8–3.0; consequently, quantification can only be performed up to a level of 2.5 ppm. In addition, the 1 ppm standard, being the lowest standard containing the analyte, returned a quite high absorbance value of 2.5. Therefore, it was decided to dilute the available standards provided with the kit in order to obtain a more suitable calibration curve. The protein concentration of the native reference sample was underestimated for the samples containing 50 and

100 ng ml⁻¹ soybean protein in a concentration-dependent manner with on average 12% (Figure 4c). Exceeding the calibration range, the reference samples containing 500, 1000 and 1500 ng ml⁻¹ soybean protein could not be considered and the latter two were not included in Figure 4(c). Upon heating without glucose for 24 h at 70°C, only about 2% of the proteins could be detected (data not shown) with an additional decrease to only 1% when extended to 48 h. When soluble wheat proteins were added to the system, the measured concentration was lower compared with the reference sample (15–20%); in contrast to the observations described for the Veratox[®] Soy Allergen, a lower signal was measured when more wheat proteins were added. Also, an almost complete loss of signal was observed after heating in the presence of soluble wheat proteins, with only 1% of the soybean proteins being detected after 24 h (data not shown) and 0.2% after 48 h. Similarly, no additional effect of adding glucose during the heat treatment could be seen.

Discussion

This study investigated different commercial ELISA kits for the detection of soy proteins and the influence of protein glycation. Application of the reference soybean protein solution in the various kits resulted in an extreme overestimation of the actual protein concentration in the Veratox Soy Allergen and BioKits Soya assays, while for the Soy Residue assay an underestimation was recorded. In addition, the amount of soybean proteins measured was dependent on the concentration in the sample applied. Similar observations have been made in a validation study of five commercial ELISA kits for peanut detection (Poms et al. 2005). A possible explanation for the differences between the actual and the measured soybean protein concentration that were obtained with the different kits could be found in the nature of the standard solutions of the respective kits. In the Soy Residue kit a single protein, namely the Soy Trypsin Inhibitor, is used as the target analyte. Possibly the factor used to convert the soy trypsin inhibitor concentration to total soy protein concentration is not the same as the actual proportion soy trypsin inhibitor present in the soybean protein extract used in this study. Strategically, the choice for a single marker protein for the detection of soybean proteins in processed food products is especially risky in terms of susceptibility to protein modification and its impact on detection, as was moreover demonstrated in this study. The high overestimation of the actual soy protein with the BioKits Soya Allergen assay demonstrate that the antibodies used to develop the assay recognise better the proteins that are present in the soybean protein extract used in this study than those present in the standard solutions

of the kit. This could be due to the use of different soybean varieties to produce the protein extract. Immunochemical activity of peanut proteins has also been shown to be dependent on the peanut variety used (De Meulenaer et al. 2005). These observations indicate that the test results depend heavily on the assay used and urge the need for reference materials for the development and validation of standardised methods.

Upon heating the reference soybean protein solution in the absence of glucose, a decreased detection was observed in the BioKits Soya Allergen, however still overestimating the actual soybean protein concentration. In contrast, both the Veratox Soy Allergen and the Soy Residue assay lacked total robustness. The complete loss of detectability can probably be due to the denaturation of soybean proteins, especially because only minor changes in terms of formation of protein carbonyls and losses of lysine residues were observed as compared with the changes observed in the model systems containing glucose (Figures 1 and 2). However, the observed loss of intensity of the *Gly m 5* allergen upon heating without glucose, indicating denaturation, might explain the lowered detection by the BioKits Soya Allergen Assay (Kitabatake et al. 1989). The *Gly m 6* allergen, with a denaturation temperature of about 90°C, could then account for the remaining detection. The Soy Trypsin Inhibitor shows a thermal transition at 60–65°C which could explain the decreased detection with the Soy Residue assay after heating at 70°C (Roychaudhuri et al. 2004). Although the denaturation of this protein has been shown to be reversible, fast cooling of the model systems, by putting the samples on ice after heat treatment, could have prevented a proper refolding.

When soybean proteins are present in a food product, protein cross-linking can occur with other (bulk) proteins, which could have an additional impact on detection. Therefore, the impact of heating soybean proteins in the presence of soluble wheat proteins was also investigated. In a previous study on hazelnut proteins it was seen that soluble wheat proteins can have an additional impact (Cucu et al. 2010). However, no additional impact on the detection of soybean proteins was seen when soluble wheat proteins were added to the model systems.

During heating of the reference soybean proteins with glucose either with or without soluble wheat proteins, changes at a molecular level took place as evidenced by a number of parameters investigated (Figures 1–3). As a consequence, these changes on protein level led to further decreases in detectability, especially for the BioKits Soya Allergen Assay. Nevertheless, in the same kit additional protein cross-linking with other soluble wheat proteins via the produced protein-bound carbonyls during glycation did not further affect the detectability of the soybean proteins. As a complete lack of robustness of

the Veratox Soy Allergen and the Soy Residue was observed upon heating soybean proteins alone, no additional impact of their heating with glucose either in the presence or absence of soluble wheat proteins could be seen.

The results show that the detection of soybean proteins by means of commercial ELISA kits is strongly influenced by processing. This implies that certain assays could falsely declare a processed food product as 'free of soybean proteins', while soybean proteins might still be present. Consequently, this holds a risk for allergic consumers. In the same time some other kits, such as BioKits Soya Allergen and Veratox Soy Allergen, show strong overestimation especially for the native soybean proteins which presents risks for the food industry where the product withdrawal from the market is based on the results obtained with similar commercial kits. In conclusion, there is still a lack of sensitive analytical methods for the detection of traces of allergens in processed foods and thus controls cannot be carried out reliably. Moreover, in this study, by inducing a Maillard reaction in soybean proteins in a buffered system, the abstraction of possible consequences of protein insolubility and eventual extractability was made. This implies that within a real food product it might be expected that estimations of the contamination levels would be even more severely affected. It should therefore be emphasised that the tested ELISA assays can be useful to give an indication of the presence of contaminants, rather than an exact quantitative value of the contamination level. But even if such assays are used in a mere qualitative context, utmost care should be given with respect to the interpretation of the results.

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