

Detection of Soy Proteins in Processed Foods: Literature Overview and New Experimental Work

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Several tests for the detection of soy proteins in foods have been described in the literature, and some are commercially available. This article gives an overview of these methods and discusses the advantages and disadvantages of each individual method. Based on the conclusions of this inventory, an experimental approach was designed to improve the sensitivity of measuring soy protein in processed foods. The aimed sensitivity is 10 ppm (10 μg soy protein in 1 g solid sample), which is over 100-fold lower than presently available tests. The aimed sensitivity is this low because levels of food allergens at 10 ppm and above may provoke reactions in food allergic persons. Native soybean meal, soy protein isolate, soy protein concentrate, and textured soy flakes were used as test materials. Several extraction procedures were compared and a new method using high pH was selected. Polyclonal antibodies were raised in rabbits and goats, and immunopurified antibodies were used in sandwich and inhibition enzyme-linked immunosorbent assay (ELISA). Extraction at pH 12 resulted in good yields for all tested samples, both quantitatively (Bradford) and qualitatively by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunopurified rabbit antibodies against this extract used in a competition ELISA format resulted in a sensitive test with a detection limit of 0.02 $\mu\text{g/mL}$, corresponding to 0.4 $\mu\text{g/g}$ (0.4 ppm) in food samples. Cross-reactivity with some main food ingredients was measured and appeared to

be negative in all cases. The presently developed test is applicable for soy ingredients and soy-containing foods that are processed in different ways. The limit of quantitation is 1 ppm, which is an enormous improvement over earlier described methods.

Soy is a food ingredient with both techno-functionality as well as bio-functionality. The beneficial effects of soy isoflavones on health have been reviewed recently (1), and food products containing 25 g soy per intake may claim to be healthy [see U.S. Food and Drug Administration (FDA) regulations at www.FDA.gov]. Soy is used in a number of applications as the consumer product diversification in the past decade(s) has required improved technological function of ingredients. In terms of authenticity and product adulteration, such as for high-quality meat products (2), methods to exclude the presence of soy protein in food products are needed. This article provides an overview of methods to detect soy proteins in food. Many methods were published in the 1980s, when the use of soy in foods expanded enormously. Test requirements then related to specificity, with respect to other food ingredients, mainly meat, and recovery for processed foods. The sensitivity was of less importance; 0.1 or 1% (w/w) of soy was a relevant detection limit. With food allergies on the increase this past decade, came the awareness that only minute amounts of soy, in particular soy protein, may threaten soy-allergic consumers. Because the minimal provoking doses of food allergens are usually low, in the μg to mg level, the sensitivity of soy tests is now an important issue. For peanut, another member of the Legumae family, thresholds for subjective reactions of 100 (3) and 30 μg (4) have been described. It is expected that the threshold for objective symptoms and more severe reactions are significantly higher (5), as demonstrated by several case reports on inadvertent intake of peanut-containing foods. For example, ingestion of approximately 10 mg peanut protein

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present in cookies (6) and 45 mg peanut protein present in soup (7) resulted in severe allergic reactions. Only limited information is available on the threshold doses of soy protein for soy allergic individuals. In fact, a multicenter study to investigate minimal provoking doses has recently started. Some case reports show that minor amounts of soy, well below 0.1 and 1% (w/w), can lead to allergic reactions (8, 9).

This article aims to review tests that have been described in literature for the detection of the different methods. In addition, we describe a new immunochemical test that we developed for the sensitive detection of soy in processed foods.

Classification of Soy Proteins

Soybeans have favorable agronomic characteristics, including a good adaptability to a wide range of soil types and an ability to fix nitrogen, which makes the soybean a good rotational crop for use together with high nitrogen consuming crops, such as corn and rice (10). Furthermore, soybeans are an economically interesting agricultural product because of their unique chemical composition. On an average dry matter basis, soybeans contain about 40% protein and 20% oil (11). With this composition, soybeans have the highest protein content among all the food crops and second highest with respect to oil content of all food legumes. The protein fraction can be divided into 3 groups: (1) proteins involved in metabolism; (2) structural proteins (ribosomal, chromosomal, and membrane proteins); and (3) storage proteins, which include approximately 80–90% of the total soybean protein. Storage proteins are globulins, which precipitate at pH 4.5–4.8 and are therefore referred to as acid-precipitable proteins (10). This acid-precipitation still forms the basis for the industrial preparation of soy protein concentrates (SPC) and soy protein isolates (SPI).

Although soy proteins have been often classified according to their sedimentation coefficients, in this article we use the nomenclature that takes into account immunological differences of the soy proteins, because that aspect is relevant for developing tests for measuring soy proteins. Immunologically, soybean globulins can be divided into glycinin, β -conglycinin, and trypsin inhibitors and some proteins that are less abundantly present. The minor proteins in soybeans include monophosphatase, phosphodiesterase, calmodulin, α -galactosidase, lactate dehydrogenase, lipoxxygenase, β -amylase, lectins (or agglutinins), polygalacturonase-inhibiting protein, metallothionein, urease, and β -glucosides (12). Our focus will be on glycinin, β -conglycinin, and the trypsin inhibitors, which represent >85% of the total protein content and are therefore a main target for detection.

Glycinin

Glycinin (11S) consists of an acidic, ca 38 kDa (kilodaltons), and a basic polypeptide, ca 20 kDa (13), linked by a single disulfide bridge, except for the acidic polypeptide A₄ (14). Each pair of acidic and basic polypeptides is encoded by a single gene and cleaved post-translationally. Generally,

multiple genes encoding for glycinin are present in each soy species (15). The pH and ionic strength influence glycinin molecular structure on tertiary and quaternary folding level. At pH 7.6 and at an ionic strength of 0.5, glycinin is mainly present in a hexameric form of 360 kDa with a sedimentation coefficient of 11S (16). Lowering the ionic strength to 0.01 at pH 7.6 causes glycinin to dissociate to a trimeric form (7S), which has a more nonstructured conformation. At lower pH (2.2–3.8), the glycinin complex further is denatured at the tertiary and secondary folding levels (17).

β -Conglycinin

β -Conglycinin (7S) consists of 3 subunit combinations of α' (57–72 kDa), α (57–68 kDa), and β (42–52 kDa), giving 7 heterogeneities, all with a molecular weight of around 180 kDa. The subunits are noncovalently associated via hydrophobic and hydrogen bonding. Furthermore, the subunits are glycosylated (5%; 18). As a function of pH and ionic strength, β -conglycinin dissociates and aggregates. At pH 7.6, the 7S form predominates at an ionic strength of 0.5 or higher, while at ionic strengths <0.2 the 9S/10S form (the dimer of the 7S form) is predominantly present. In the intermediate ionic strength region, varying amounts of monomer and dimer are present. The protein exists as a monomer at about pH 3, but associates to form a dimer at or above pH 6. At intermediate pH, the protein exists as a mixture of monomers and dimers. At pH 2 and low ionic strength, β -conglycinin is reversibly converted into smaller (monomeric) forms (19).

Protease Inhibitors

Protease inhibitors that have been isolated from soybeans are the Kunitz trypsin inhibitor and the Bowman-Birk inhibitor. The Kunitz trypsin inhibitor has a molecular weight between 20 and 25 kDa, 2 disulfide bonds, and a specificity directed primarily towards trypsin. The Bowman-Birk inhibitor has a molecular weight of about 8 kDa and has 7 disulfide bonds. It can inhibit both trypsin and chymotrypsin at independent reactive sites (10). In contrast to glycinin and β -conglycinin, no higher-order forms of protease inhibitors are present in soy.

Influence of Denaturation on Soy Protein Solubility

Foods that contain soy proteins are usually subjected to some form of heat treatment (e.g., pasteurization, sterilization) during processing to obtain a certain shelf life, an inactivation of the antinutritional properties, and certain functional properties. Proteins generally aggregate as a result of heat denaturation, and this is also observed for soy proteins. Solubility data on soy proteins are plentiful, but their value for obtaining an overview is frequently limited because of the large variation in heat treatment given, soybean variety used, conditions of determination (pH, ionic strength), solvent used, etc. These factors all affect apparent solubility (20).

Glycinin

The temperature at which glycinin denatures depends on ionic strength (I) and pH conditions. At I = 0.5 and pH 7.6, glycinin denatures at 94°C. When the ionic strength and pH are lowered, the denaturation temperature decreases. At conditions where glycinin occurs both in an 11S form and as an 7S form, a 2-step denaturation takes place in which the 7S form is linked to the lowest denaturation temperature (21, 22). Koshiyama et al. (23) and Hashizume et al. (24) also found that at pH 7.6 the heat denaturation temperature of glycinin was influenced by ionic strength. Koshiyama et al. (23) found no indications for a correlation between the denaturation temperature and the protein structure at ambient temperatures. Furthermore, according to Yamagishi et al. (25) and Hashizume and Watanabe (26), at I = 0.5 and pH 7.6 the acidic polypeptides were present in the soluble fraction after heat treatment, while the basic polypeptides were found in the precipitate, as was also found by Lakemond et al. (22) at lower ionic strengths. At pH 5.2 and 3.8, all glycinin precipitated after heat treatment except under the conditions of I = 0.03 and pH 3.8 (72% soluble; 22).

β -Conglycinin

It is generally believed that β -conglycinin denatures at lower temperatures than does glycinin. The denaturation temperature of β -conglycinin at pH 7.0 is increased from 77° to 100°C upon increasing the salt concentration from 0.05 to 2.0M (20). Thermal denaturation of β -conglycinin is also sensitive to changes in pH (27).

β -conglycinin that is dissolved in distilled water (0.5%, w/w, pH 7.5) can be heated without developing turbidity even at 100°C for 30 min. Upon increasing the ionic strength, β -conglycinin does not stay soluble. During heating, β -conglycinin dissociates into its subunits and, based on the ionic strength, aggregation can take place afterwards (27).

Trypsin Inhibitors

Purified Kunitz trypsin inhibitor lost most of its activity at pH 7.0 after 5 h of thermal denaturation at 100°C. Bowman-Birk inhibitor retained over 75% of its antitryptic activity after 5 h heat treatment (28). Sorgentini and Wagner (29) found that Kunitz trypsin inhibitor denatures in water around 76°C and that Bowman-Birk denatures around 77°C. These temperatures are slightly affected by ionic strength. Most studies performed on heat denaturation of trypsin inhibitors focus on the remaining activity of the inhibitors after heating, but no information is present about the effect of heating on solubility and structural aspects.

Total Protein Fraction

Denaturation, aggregation, and precipitation characteristics of purified fractions of individual proteins can be different from those of the same soy proteins heated within a soy or food matrix. For example, the soybean protease inhibitors within a crude extract of raw soy flour lose their activities at pH 7.0 more rapidly than do inhibitors in pure

form (28). Additionally, still-active trypsin inhibitors were found after toasting and especially extrusion of soybean meal, suggesting that the trypsin inhibitors were embedded in the protein matrix and were thereby protected against heat inactivation (30). The pH and ionic strength of the aqueous solvent have a significant effect on solubility behavior of soy proteins (20), and we re-investigated this (Figure 1). In water and 0.2M NaCl, SPI had a low solubility between pH 4 and 5. In 0.5M NaCl, SPI also had a low solubility, but the actual minimum was observed at pH values <3. Heating did not affect solubility in the absence of NaCl. At 0.2M NaCl solubility decreased 20–30% at pH values >5, and at 0.5M NaCl it decreased 10–30% at pH 3–7. For β -conglycinin >pH 5, the amount of dissolved protein decreased with increasing salt concentration. Furthermore at 0.2 and 0.5M NaCl, the soluble fraction contained mainly acidic polypeptides of glycinin, whereas at 0M NaCl, equal amounts of acidic and basic polypeptides were found. At pH 2 and 3, glycinin at I = 0.2 and 0.5 some of the glycinin protein was soluble as was β -conglycinin.

Extraction of Soy Protein from Food Products

The significant differences in solubility between the various commercially available soy protein ingredients can be explained by the pretreatment sustained by the ingredients. The protein solubility at pH 7.6 in 8M urea and 0.1M 2-mercaptoethanol (31) or 0.01% dithiothreitol (DTT; 31) of SPI after extrusion has been studied. Prudencio Ferreira and Areas (31) found that solubility was 100% in these buffers, whereas Marsman et al. (30) found that 15–30% of the protein fraction still remained insoluble. Henn and Netto (32) found that the protein dispersibility index (PDI) values of commercial soybean isolates varied from 11.7 to 88.7%.

Most of the studies performed on extraction of soy proteins from food products have focused on meat products (33–42). In 1976, a workshop, organized on the identification of protein additives or substitutes in meat products and human foodstuff, resulted in 22 papers that were published in *Annales de la Nutrition et de l'Alimentation* in 1977 (43). The majority of these methods required soluble soy protein rather than solid or dispersed food products; therefore, extraction of soy proteins is of key importance.

Several methods have been used for extraction of soy proteins. It is known now from these previous studies that it is hardly predictable what protein fraction stays soluble after heat denaturation. Extraction with a hot solution of 8M urea/SDS in combination with reducing agents such as DTT/2-mercaptoethanol is performed often because it resolubilizes precipitated protein (30, 31, 35, 36, 40, 44, 45). The commercially available soy protein assay also uses this principle. Agater et al. (41) used enzymatic hydrolysis with trypsin after heat treatments to solubilize the soy protein. Furthermore, it is known that alkali treatment usually improves the solubility of soy proteins, particularly if the pH exceeds 10.5, by causing dissociation and disaggregation of the soy proteins (20). Kamm (33), for example, used a 2%

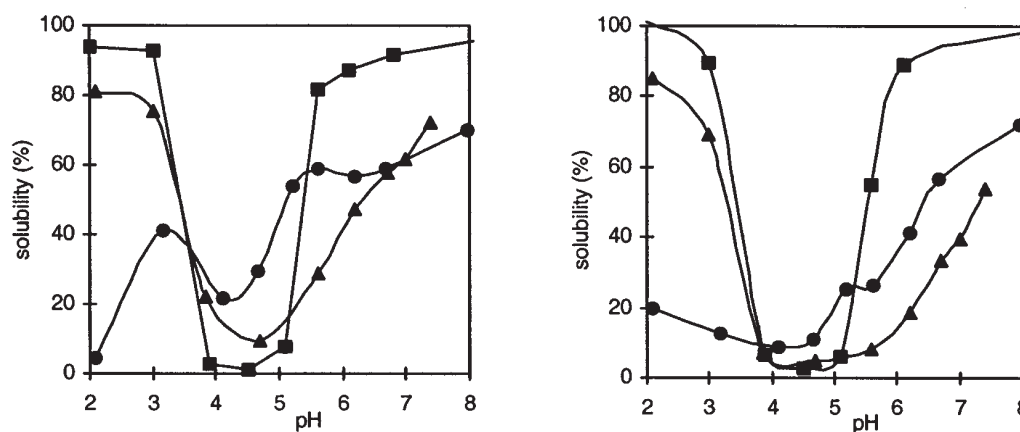


Figure 1. Solubility of unheated (left) and heated (right) 1% soy protein dispersions as a function of pH at added salt concentrations of 0 (■), 0.2 (▲), and 0.5M NaCl (●).

sodium carbonate buffer at pH 8.5 (in a blender) to extract the protein. Medina (42) used a sodium carbonate buffer at pH 9.8 (blender and sonication). Koers and Steinhart (38) used 10mM Tris HCl buffer, pH 9.0, with 1.5% *N*-acetyl-*N,N,N*-trimethylammoniumbromide (CTAB), 3M urea, 6% glycerol, and 0.01% DTT (using an Ultra Turrax, Ika, Staufen, Germany). This group claimed that CTAB is not as denaturing as SDS and thereby allows separation of proteins that are virtually unchanged from their naturally occurring state.

It is not possible to discuss the efficiency of the different types of methods to extract soy protein from a food matrix because of scant data in the literature.

Detection of Soy Proteins

Many different approaches for detection of soy proteins and other soy components in meat have been reported and reviewed (43, 45). All tests were developed for measuring comparatively larger quantities of soy protein (percents of total weight) in food products to detect the use of soy as a protein fortifier/binder. Only a small number of tests detect traces of soy cross-contamination that threaten soy-allergic consumers. These are mainly immunochemical tests based on antibody-antigen interactions and are usually more sensitive than chemical or biochemical methods. The different techniques used for soy detection are summarized below.

Requirements for Sensitivity

With regard to the safety of foods for soy-allergic consumers, the sensitivity of the detection method is of utmost importance. It is clear that concentrations well below 0.01% (100 ppm) can cause allergic reactions. For peanut, another member of the Legumae family, minimal doses that elicited subjective reactions of 30 (4) and 100 μ g (3) have been described. This corresponds to 0.3 to 1 ppm when a serving size of 100 g is considered. For soy, no accurate data exist on minimal provoking doses. Based on threshold levels of other food allergens, and taking into account case reports, Taylor

and Nordlee (46) postulated that a detection limit of 10 ppm is a useful figure. However, because data on soy are very limited, a recommended detection limit is 1–2 ppm. Four commercial kits are available for peanut, all with detection limits between 1 and 10 ppm. This range should also be the target for other new allergen protein tests.

Detection of Soy Protein Using Nonantibody-Based Tests

Electrophoresis can be used for analyzing soy proteins only when soy is the main ingredient because proteins from other sources (e.g., meat) are also visualized with this method. An advantage is that electrophoresis of proteins in gels containing detergent or urea is easy to perform. The reliability of the data usually depends on solubilization and disaggregation of the proteins and on quantification by dye binding and scanning. The resolution of complex mixtures from heated or canned products is often poor (45) and therefore not adequate for detection of soy proteins in complex food matrixes.

Specific cleavage of the protein (e.g., by trypsin) yields a complex mixture of soluble peptides of which a characteristic peak can be selected after fractionation as a measure of the parent foreign proteins. Yasumoto et al. (47) used a heat-stable pentapeptide, SP 1, which is derived from a basic subunit of glycinin after tryptic hydrolysis, and was detected by ELISA. In principal, this method is interesting because hydrolysis is also an effective way to solubilize the protein (47–49). However, this method is laborious and not easily used for routine work. It is also possible to determine the amino acid composition from total hydrolysates. Computer-assisted comparisons with data from known mixtures lead to estimates of the ratios of proteins present, provided that they are not too similar in amino acid composition (45). The sensitivity of this method is not sufficient to detect traces of soy in processed foods. As for electrophoresis, proteins from sources other than soy may complicate the analysis.

Detection of Soy Components Other Than Protein; Indirect Analysis

Because direct protein measurement is sometimes difficult due to solubility issues, indirect methods based on appropriate nonprotein markers associated with soy may be useful. Suitable markers have been chosen for their stability and ease of assay under the processing conditions used. Examples of markers that have been used for assay development include insoluble polysaccharides, oligosaccharides, protein-bound sugars, free amino acids, free peptides, phytate, saponins, sterols, and metals (45). Another approach used histology; a microscopic examination of calcium oxalate crystals and plant cell components indicated the presence of soy. Staining for cell wall polysaccharides could also be useful in this respect (45). The detection level of this method is too high for quantification of traces of soy.

One method of particular interest is based on the presence of polyphenols. Mellenthin and Galensa (37) extracted soy polyphenols from meat products and analyzed them with capillary electrophoresis and liquid chromatography (LC). This method is based on the detection of naturally occurring polyphenols within legumes such as soy, lupin, peanut, fenugreek, lentil, and pea. The aglycons genistein and daidzein and their corresponding glycosides were suitable indicators for soy protein addition. They not only occurred in all soybean species but also in all individual SPIs and SPCs (50). Even from complex matrixes, isoflavones were extracted in 5 min with an extraction solvent consisting of acetonitrile and water (37). Additives of SPIs up to 0.01–0.1% (100–1000 ppm) could be detected in meat products. The method was also suitable for other matrixes such as cheese, cakes, waffles, pastries, and soy fats. In a vegetable matrix, other polyphenols can often interfere with detection of isoflavone metabolites (37). The disadvantage of this method is that it requires specialized equipment to perform the test.

More recent public discussion on genetically modified organisms (GMO) and foods originating from such crops led to the development of assays for GMO soy, with a reasonable sensitivity [0.9%; European Union (EU) legislation; for more information, see http://europa.eu.int/comm/food/fs/gmo/gmo_ongoing_en.html]. Methods based on DNA, most often polymerase chain reaction (PCR) tests, are applied to distinguish between wild-type and GMO soy. As a test control, a gene present in wild-type soy is detected as well. Van Duijn et al. (51) for example, measured the gene of soy lectin as a positive control. This test has also been used to detect soy in processed food with a reported detection limit of 0.01% (100 ppm).

The sensitivities of the described indirect methods are between 0.01 and 1% (100–10 000 ppm). These are appropriate for detecting soy added as a functional ingredient, but not for detecting traces originating from cross-contamination. Besides this practical disadvantage, there is a theoretical concern. In the attempt to protect soy-allergic consumers, the allergic component, i.e., protein, should be measured rather than a marker. This is of key

importance, because industrial soy processing results in a variety of soy protein products that may have lost other (marker) components during processing.

Antibody-Based Tests

ELISA is a powerful analysis tool for detection of specific proteins. It has the advantage of simultaneously conducting a larger series of samples at a high level of sensitivity. A large number of investigators have used ELISA methods to detect soy protein in meat products. Furthermore, ELISAs can be used to follow conformational changes of specific soy proteins. A wide variety of antibodies have been raised against native or denatured (subunits/polypeptides of) glycinin, β -conglycinin, Bowman-Birk inhibitor, Kunitz trypsin inhibitor, etc. Immunochemical methods are generally limited to the qualitative screening of raw or mildly processed products, because protein denaturation often alters the antigen-antibody interactions (45). This has been studied by different authors (26, 45, 52–61), and the most important results are discussed below.

Antibodies directly against soy can be raised in laboratory animals, usually rabbits, mice, goats, sheep, or donkeys. Individual animals may react differently to immunization protocols, and it is difficult to predict which species is the best host for raising antibodies against soy. Another issue influences the success of raising antibodies against soy. Soybean protein is a common substance in animal food; therefore, the antigenic responses obtained after immunization are not expected to be high. This may lead to antibodies with low affinity, and, consequently, ELISAs constructed with such antibodies have poor sensitivities. Brandon et al. (55) also encountered this problem when raising antibodies against Bowman-Birk inhibitor. They solved the problem by immunizing mice that were fed soy-free diets.

Once antibodies have been raised, several methods can help to improve ELISA sensitivity. Plumb et al. (58) found that the ELISA format can influence detectable immunoreactivity. Some soy flours with very high reactivity in a 2-site ELISA displayed no reactivity in a direct ELISA. Most soy protein ELISA methods have not been developed to obtain maximum sensitivity. Thus, the method itself could be optimized, as is described by Chidro et al. (62), who reached a detection limit of 1 ng/mL for the quantification of prolamines in food. Furthermore, the choice of the target protein is important. Proteins that are less abundantly present in soy will be more difficult to detect, and, consequently, the detection limit will be high. Therefore, it seems logical to choose glycinin or β -conglycinin as an antigenic protein since these are the major soy proteins. Whole soy protein may be a good alternative. One should keep in mind that allergens may be hindered in interacting with antibodies because of heat-induced aggregation, resulting in a decrease in immunoreactivity (63); this can adversely affect the recognition of soy proteins in immunoassays.

Antibodies Against Glycinin

Ravenstein and Driedonks (36) raised antibodies against the SDS-denatured acidic polypeptides of glycinin in rabbits. An ELISA constructed with these antibodies had a detection limit of 0.5% (5000 ppm). Applying the antibodies in an immunoblotting experiment decreased the detection limit enormously, but this was not considered relevant because soy is usually used in concentrations at 1% and higher. Meisel (57) immunized chickens with SDS-denatured glycinin and obtained specific IgY antibodies from egg yolk. With these antibodies, an ELISA with a sensitivity range between 0.5 and 256 µg/mL for dilutions of SPI was constructed. The detection limit was 0.5 µg/mL, corresponding with 0.1% soy protein spiked in a food matrix. IgY antibodies used in immunoblotting even allowed the detection of the acidic polypeptide of glycinin at nanogram levels (57).

Plumb et al. (58) raised antibodies against native glycinin. They found that heating glycinin at pH 7.6 caused the immunoreactivity to decrease to around 50% of the original value but increased sharply at temperatures above 92°C. These results are in agreement with Demonte et al. (60) but differ from the results of Iwabuchi and Yamauchi (53), who found that the antigenic activity disappeared after heating. Huang et al. (61) found that the epitope identified by Plumb et al. (58) corresponds to residues 86–104 of the acidic polypeptides of glycinin A1aB1b and lies on the C-terminus of the proteolytic intermediate known as glycinin-T. The epitope seems to be continuous in nature. Iwabuchi and Yamauchi (53) studied the effect of ionic strength on thermal denaturation of soybean glycinin. No effects of ionic strength were found up to $I = 0.7$. A reduction in immunogenicity also occurred when glycinin was taken to pH 2.0 and 11.0 and exposed to high temperatures (52).

Antibodies Against β -Conglycinin

Plumb et al. (59) raised antibodies that were specific for the native α and α' subunits of β -conglycinin. β -conglycinin immunoreactivity was increased as the protein was heated, reaching a maximum at the denaturation temperature of 65°C. This phenomenon is unusual as most thermally denatured proteins have low immunoreactivity when probed with antibodies raised against native protein. This was also found at pH 7.6 at different ionic strengths (27, 52). The epitope of the antibody used by these researchers corresponds to the residues 78–84 in the acidic extension present in the α' subunit of β -conglycinin (59) and seems to be continuous in nature (61). A linear epitope, in contrast to a conformational epitope, is expected to be more heat-stable and may become more exposed after denaturation. This may explain the increased detectability of heated β -conglycinin.

Antibodies Against Trypsin Inhibitors

Brandon et al. (54) worked with antibodies against native and heat-denatured soybean Kunitz trypsin inhibitor. Antibodies raised against the denatured inhibitor were specific for the denatured conformation of the protein. In

contrast, the native inhibitor elicited antibodies that selectively recognized determinants in both native and heat-treated proteins. Barkholt et al. (56) raised an antibody against Kunitz soybean trypsin inhibitor that worked equally well against native and denatured protein. Brandon et al. (55) used antibodies that bind the Bowman-Birk protease inhibitor. They identified an epitope that was destroyed by heat and developed an ELISA for specific recognition of native Bowman-Birk inhibitor in the presence of denatured forms. None of these tests were optimized for sensitivity.

Antibodies Against Whole Soy Protein

Several methods use antibodies raised not against one particular protein but against the whole soy protein fraction. For example, Janssen et al. (34) detected soy proteins in meat products up to 0.1% by gel electrophoresis followed by blotting and dot-blot. All major soy fractions were recognized by the antibody. Hitchcock et al. (45) raised antibodies against "soy protein," which were "renatured" from a hot urea solution by removing or diluting the denaturant. This method could recognize heat-treated soy fractions as well as native ones. Some specialized products, however, gave little or no response, presumably because the proteins added were hydrolyzed. The method of Hitchcock et al. (45) could also be used with commercially available anti-soy antibodies (64), and a commercially available soy protein assay kit was developed. The antibody that is used is not specified in the manual. This test is meant for quantitation of soy protein levels between 1 and 10% of total weight of raw or processed mixed meat products. The relatively high detection limit is partially caused by the fact that the extracted samples must be diluted in order to prepare an environment that is compatible with ELISA. In this case, the required dilution factor was 20.

Original Work on Soy Protein Extraction and Detection

A New Method for Extraction of Soy Protein from Processed Soy Ingredients

Based on the literature on soy protein extraction, and taking into account the requirements for the new tests, soy proteins were extracted at extremely high pH (pH 12). A quantitative analysis [the Bradford method (65)] and a qualitative analysis via SDS-PAGE were performed to evaluate the obtained extracts. A set of commercially available soy ingredients that were processed in different ways was used as analytes. Table 1 shows a brief ingredient description and the protein concentrations as determined by the Bradford method.

It is clear from Table 1 that the native extraction method (Tris, pH 8.2) results in low average yield, with particular low yield for some SPCs. The urea/DTT method used in the commercially available soy test results in a higher average yield of extractable proteins. Our new method that applies pH 12 results in the highest average yield. In addition to the yield, it is also important to examine the nature of the extracted proteins because the analyte should not be distorted

Table 1. Extractability of processed soy ingredients using 3 different methods

Sample description	Type	Gel code	Extraction recovery, % related to product specification		
			Tris, pH 8.2	Urea/DTT	pH 12
Soy 200/90 (own supply) light toasted	Meal	1	39	52	67
Central Soya Centex 4220 Textured	Flour	2	6	46	45
Central Soya Pro Fam 981	Isolate	3	39	43	60
ADM Pro Fam 891	Isolate	4	33	46	51
ADM Pro Fam 781	Isolate	5	28	36	37
Central Soya Pro Fam 646	Isolate	6	28	46	66
ADM Arcon S	Concentrate	7	34	47	64
ADM Arcon SM 066-405	Concentrate	8	41	50	67
ADM Arcon F	Concentrate	9	3	48	52
Central Soya Response 4400 Textured	Concentrate	10	5	35	52
Central Soya Procon 2000	Concentrate	11	4	48	59
Central Soya Promine DS Functional	Concentrate	12	3	45	42
ADM Arcon T	Flakes	13	ND ^a	ND	ND
Mean (±SD)			20 (±15)	45 (±5)	55 (±10)

^a ND = Not determined.

for adequate measurement. Each extract as prepared for Table 1 was analyzed on SDS-PAGE, and the results are shown in Figure 2. When the native method (Tris, pH 8.2, panel A) is compared with both the urea/DTT and pH 12 method, it is clear that a number of processed ingredients do not show clear protein bands on SDS-PAGE. In fact, these particular samples resemble to a large extent the samples with a low quantitative extraction yield (Table 1). A comparison of the urea/DTT method with the pH 12 method shows that each sample is recovered to a similar extent. The protein bands around 46 kDa are more pronounced with the urea/DTT method, whereas the pH 12 method results in more resolution for the recovered protein bands. This indicates that extraction at pH 12 re dissolves protein aggregates, especially in the case of SPCs. The high pH does not affect the primary sequence of the individual protein bands, since no degradation by hydrolysis is observed on the gel. Taken together, the pH 12 method resulted in the highest yields (Table 1) and the sharpest protein bands on the gel (Figure 2) and is judged somewhat better than the urea/DTT method that is recommended for the commercially available test.

Raising Antibodies

The extracts prepared with our new method at pH 12 contained a representative amount and pattern of soy proteins, regardless of the ingredient pretreatment. A mixture of SPI, SPC, and texturized soy was extracted at pH 12, and this extract was used for raising antibodies in goats and rabbits. A classical protocol for immunization was used, and prior to that, animals were kept on a soy-free diet for at least 3 months. Sera were collected and tested for the presence of antibodies

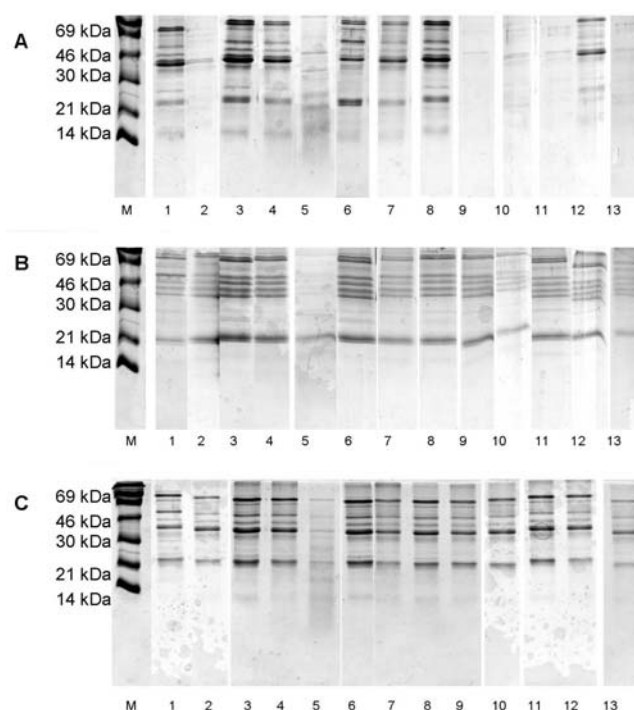


Figure 2. Qualitative analysis of soy extracts prepared with different extraction methods. Panel A: extraction with Tris, pH 8.2. Panel B: extraction with urea/DTT. Panel C: extraction with pH 12. Markers are at left lane of each gel (M) and molecular weights are indicated in left margin.

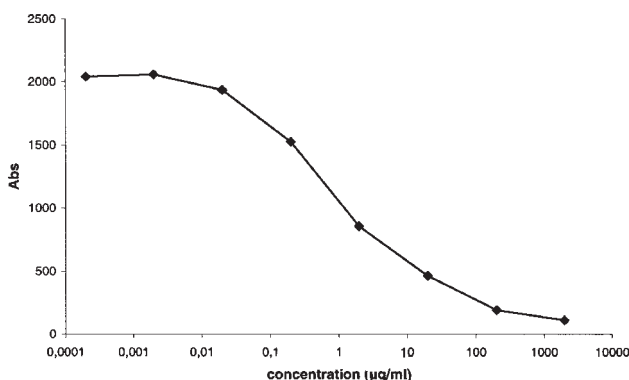


Figure 3. Standard curve for soy protein ELISA. ELISA plates were coated with pH 12-extracted soy and incubated with mixtures of rabbit antibody against pH 12-extracted soy with calibrator (pH 12-extract of soy meal).

against pH 12-extracted soy protein in a direct ELISA. Sera with titers >10,000 were used for assay development. A number of ELISA formats were evaluated, including sandwich ELISA and inhibition ELISA. The best results were obtained with an inhibition ELISA using rabbit antibodies. Plates were coated with the soy-pH 12 extract and, after blocking, incubated with a mixture of the test sample with diluted rabbit antibodies. Bound rabbit antibody was detected by an antibody directed against rabbit IgG raised in goats and conjugated with peroxidase (Nordic, Tilburg, The Netherlands). A calibration curve was constructed with dilutions of an extract of untreated soy meal (Figure 3). The sensitivity range was between 0.01 and 10 µg/mL, and the detection limit was 0.02 µg/mL, which corresponds to 0.4 ppm for solid food samples.

Because it is known that soy processing influences detectability of soy proteins to a large extent, we tested a variety of soy ingredients and calculated the recovery. Table 2 shows that the recovery of our test was high (mean 73%) and that the variation between individual samples was only moderate (35%). One of the main applications of this test will be the quality control of ingredients, and other plant proteins will be often checked for the presence of traces of soy. We tested whether corn and wheat cross-reacted in this new test by extracting them and testing them in several dilutions (1 in 2 up to 1 in 2 000 000). Only the lowest dilution (2-fold) of wheat showed a minor response in our test (<10 % of the maximal signal), whereas corn did not interfere at all. The minor cross-reactivity of wheat was overcome by diluting wheat-containing samples 10-fold. This resulted in an increase of the detection limit of 10-fold, to 4 ppm, which still met our goal of 10 ppm.

Conclusions

In the past 2 decades, a vast number of tests for the detection of soy in processed food have been developed. Most of the work was done in the 1980s and focused on the detection of gross amounts of soy added to food products as a nutritional fortifier or functional ingredient. Therefore, detection limits around 0.1% (1000 ppm) were useful. Most tests are based on the immunochemical detection of proteins, such as storage proteins and protease inhibitors, that are present abundantly in soy beans. The limited solubility of soy protein after processing, i.e., heat treatment, hampered reliable soy detection until an extraction method was developed that denatured the aggregated soy. An ELISA test based on this extraction method became commercially available about 10 years ago and is now often used for detecting soy protein in meat products in cases where product adulteration is suspected. Unfortunately, this test has a limited sensitivity because the denaturing extraction buffer must be diluted to make the extract compatible with the ELISA environment. Another aspect that may have limited the sensitivity of ELISA tests in the past is the fact that virtually all laboratory animals are fed with soy-containing diets, and this may induce a tolerance for soy proteins and result in only poor anti-soy titers after immunization or antibodies with a limited affinity.

We developed a novel extraction method that uses an extraordinarily high pH of 12. This high pH resulted in a clear resolubilization, and proteolysis of the individual proteins and sub-units was not observed. It is a well-studied phenomenon that soy ingredient processing and food processing influence soy protein solubility. Our method for extraction extracts from each tested product a representative amount of protein, with an SDS-PAGE band pattern comparable with that of nontreated soy. However, some soy ingredients showed lower extractability. The average extraction yield was $55 \pm 10\%$, with a range of 37–67%. This is somewhat better than the previously used denaturing extraction procedures (urea and DTT). For true quantitation of soy protein in food, a full recovery of extraction is a prerequisite. Unfortunately, this is utopia, since the extraction must be compatible with the assay environment. The most important aspect when dealing with soy detection for allergenic residue concerns is that all ingredients are recognized to a reasonable extent. In that way, products containing traces of soy that are processed under extreme conditions, like extrusion, are also identified. A main advantage of extracting soy proteins at pH 12 was that a 2-fold dilution was sufficient to reach an acceptable buffer composition for ELISA. Using laboratory animals that were kept on soy-free diets, we could raise anti-sera with high titers, allowing for sensitive measurement when applied in inhibition ELISA. Although some work needs to be done in the validation of this assay, the results indicate that the sensitivity of our assay, around 1 ppm, combined with the minimal cross-reactivity towards other plant protein, forms a promising basis for a new soy ELISA method. Our test is sensitive enough to detect traces of soy protein that are

Table 2. Recovery of soybean detection

Type of soy	Gel code	Percent measured soy protein ^a	Listed on product specification sheet	Recovery, %
Meal	1	Used as standard	— ^b	—
Flour	2	35	54	65
Isolate	3	87	90	97
Isolate	4	79	90	88
Isolate	5	27	90	30
Isolate	6	123	90	136
Concentrate	7	61	72	86
Concentrate	8	83	70	119
Concentrate	9	25	69	37
Concentrate	10	39	65	60
Concentrate	11	35	68	52
Concentrate	12	20	68	29
Flakes	13	36	Not specified	
Mean (±SD)				73 (±35)

^a Protein concentration measured by Bradford method (65).

^b — = Not determined.

thought to provoke allergic reactions in soy-allergic consumers. A number of cases of severe allergic reactions towards soy have been described in the literature (8, 9, 66), and doses of several milligrams appeared to be enough to trigger these reactions. Although without full insight to minimal provoking doses, it is likely that test sensitivity in the low ppm range is sufficient to adequately assess the possible presence of traces of soy with regard to the safety of the soy-allergic consumer.

We conclude that our new soy protein test is sensitive and applicable for ingredients that have undergone various harsh processing procedures. This makes our test a good candidate for routine analysis by the food industry for traces of soy in foods for the protection of soy-allergic consumers. It will likely also be helpful to regulatory agencies in assessing good manufacturing practices and investigating soy-allergic consumer complaints.

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References

- (1) Fitzpatrick, L.A. (2003) *Maturitas* **44 Suppl. 1**, S21–S29
- (2) Hoogenkamp, H. (1992) *Vegetable Protein*, 2nd Ed., PTI International, Protein Technologies International, Corby, UK
- (3) Hourihane, J.O., Kilburn, S.A., Nordlee, J.A., Hefle, S.L., Taylor, S.L., & Warner, J.O. (1997) *J. Allergy Clin. Immunol.* **100**, 596–600
- (4) Wensing, M., Penninks, A.H., Hefle, S.L., Koppelman, S.J., Bruijnzeel-Koomen, C.A., & Knulst, A.C. (2002) *J. Allergy Clin. Immunol.* **110**, 915–920
- (5) Sampson, H.A. (1990) *J. Allergy Clin. Immunol.* **86**, 1–3
- (6) Kemp, S.F., & Lockey, R.F. (1996) *J. Am. Med. Assoc.* **275**, 1636–1637
- (7) McKenna, C., & Klontz, K.C. (1997) *Ann. Allergy Asthma Immunol.* **79**, 234–236
- (8) Foucard, T., & Malmheden-Yman, I. (2001) *Pediatr. Allergy Immunol.* **12 Suppl. 14**, 97–101
- (9) Foucard, T., Edberg, U., & Malmheden Yman, I. (1997) *Lakartidningen* **94**, 2635–2638
- (10) Liu, K. (1997) *Soybeans; Chemistry, Technology and Utilization*, 1st Ed., International Thomson Publishing, New York, NY
- (11) Perkins, E. (1995) in *Practical Handbook of Soy Processing and Utilization*, D.R. Erickson (Ed.), American Oil Chemists' Society Press, Champaign, IL, pp 1–28
- (12) Garcia, M.C., Torre, M., Marina, M.L., & Laborda, F. (1997) *Crit. Rev. Food Sci. Nutr.* **37**, 361–391
- (13) Staswick, P.E., Hermodson, M.A., & Nielsen, N.C. (1981) *J. Biol. Chem.* **256**, 8752–8755
- (14) Staswick, P.E., Hermodson, M.A., & Nielsen, N.C. (1984) *J. Biol. Chem.* **259**, 13431–13435

- (15) Tumer, N.E., Thanh, V.H., & Nielsen, N.C. (1981) *J. Biol. Chem.* **256**, 8756–8760
- (16) Badley, R.A., Atkinson, D., Hauser, H., Oldani, D., Green, J.P., & Stubb, J.M. (1975) *Biochim. Biophys. Acta* **412**, 214–228
- (17) Koshiyama, I. (1972) *Int. J. Pept. Protein Res.* **4**, 167–176
- (18) Yamauchi, F., Yamagishi, T., & Iwabuchi, S. (1991) *Food Rev. Int.* **7**, 281–322
- (19) Kilara, A., & Sharkasi, T.Y. (1986) *Crit. Rev. Food Sci. Nutr.* **23**, 323–395
- (20) Kinsella, J.E. (1979) *J. Am. Oil Chem. Soc.* **56**, 242–256
- (21) Schwenke, K.D., Grinberg, V.Y., Danilenko, A.N., Burova, T.V., & Tolstoguzov, V.B. (1987) *Nahrung* **31**, 183–184
- (22) Lakemond, C.M., de Jongh, H.H.J., Hessing, M., Gruppen, H., & Voragen, A.G. (2000) *J. Agric. Food Chem.* **48**, 1991–1995
- (23) Koshiyama, I., Hamano, M., & Fukushima, D. (1981) *Food Chem.* **6**, 309–322
- (24) Hashizume, K., Nakamura, N., & Watanabe, T. (1979) *Agric. Biol. Chem.* **39**, 1339–1347
- (25) Yamagishi, T., Takahashi, N., & Yamauchi, F. (1987) *Cereal Chem.* **64**, 207–212
- (26) Hashizume, K., & Watanabe, T. (1979) *Agric. Biol. Chem.* **43**, 683–690
- (27) Iwabuchi, S., & Shibasaki, K. (1981) *Agric. Biol. Chem.* **45**, 1365–1371
- (28) DiPietro, C.M., & Liener, I.E. (1989) *J. Agric. Food Chem.* **37**, 39–44
- (29) Sorgentini, D.A., & Wagner, J.R. (1999) *J. Food Biochem.* **23**, 489–507
- (30) Marsman, G.J.P., Gruppen, H., de Groot, J., & Voragen, A.G. (1998) *J. Agric. Food Chem.* **46**, 2770–2777
- (31) Prudencio Ferreira, S.H., & Areas, J.A. (1993) *J. Food Sci.* **58**, 378–381
- (32) Henn, R.L., & Netto, F.M. (1998) *J. Agric. Food Chem.* **46**, 3009–3105
- (33) Kamm, L. (1970) *J. Assoc. Off. Anal. Chem.* **53**, 1248–1252
- (34) Janssen, F.W., Voortman, G., & de Baaij, J.A. (1986) *Z. Lebensm. Forsch.* **182**, 479–483
- (35) Janssen, F.W., Voortman, G., & Baaij, J.A. (1987) *J. Agric. Food Chem.* **35**, 563–567
- (36) Ravenstein, P., & Driedonks, R.A. (1986) *J. Food Technol.* **21**, 19–32
- (37) Mellenthin, O., & Galensa, R. (1999) *J. Agric. Food Chem.* **47**, 594–602
- (38) Koers, M., & Steinhart, H. (1997) *Z. Lebensm. Forsch.* **205**, 224–226
- (39) Guenter, H. (1969) *Arch. Lebensmittelhyg.* **20**, 97–106
- (40) Molander, E. (1982) *Z. Lebensm. Forsch.* **174**, 278–281
- (41) Agater, I.B., Briant, K.J., Llewellyn, J.W., Sawyer, R., Bailey, F.J., & Hitchcock, C.H.S. (1986) *J. Sci. Food Agric.* **37**, 317–331
- (42) Medina, M.B. (1988) *J. Agric. Food Chem.* **36**, 766–771
- (43) Daussant, J., & Massau, J.P. (1977) *Annales de la Nutrition et de l'Alimentation* **31**, 139–270
- (44) Parsons, A.L., & Lawry, R.A. (1977) *Annales de la Nutrition et de l'Alimentation* **31**, 201–205
- (45) Hitchcock, C.H.S., Bailey, F.J., Crimes, A.A., Dean, D.A.G., & Davis, P.J. (1981) *J. Sci. Food Agric.* **32**, 157–165
- (46) Taylor, S.L., & Nordlee, J.A. (1996) *Food Technol.* **50**, 231–238
- (47) Yasumoto, K., Sudo, M., & Suzuki, T. (1990) *J. Sci. Food Agric.* **50**, 377–389
- (48) Yasumoto, K., Watsui, T., Sudo, M., Todoroki, H., & Suzuki, T. (1985) *Nutr. Sci. Soy Protein* **7**, 18–21
- (49) Yasumoto, K., Todoroki, H., & Suzuki, T. (1985) *Nutr. Sci. Soy Protein* **6**, 21–27
- (50) Wang, H., & Murphy, P.A. (1994) *J. Agric. Food Chem.* **42**, 1666–1673
- (51) van Duijn, G.J., van Biert, R., Bleeker-Marcelis, H., van Boeijen, I., Adan, A.J., Jhakrie, S., & Hessing, M. (2002) *J. AOAC Int.* **85**, 787–791
- (52) Iwabuchi, S., & Shibasaki, K. (1981) *Agric. Biol. Chem.* **45**, 285–292
- (53) Iwabuchi, S., & Yamauchi, F. (1984) *J. Food Sci.* **49**, 1289–1294
- (54) Brandon, D.L., Bates, A.H., & Friedman, M. (1988) *J. Food Sci.* **53**, 102–106
- (55) Brandon, D.L., Bates, A.H., & Friedman, M. (1989) *J. Agric. Food Chem.* **37**, 1192–1196
- (56) Barkholt, V., Ebbelohj, K., Sorensen, S., Arentsen, C., Dahl, M., & Frokier, H. (1994) *Food Agric. Immunol.* **6**, 163–166
- (57) Meisel, H. (1993) *Int. Dairy J.* **3**, 161
- (58) Plumb, G.W., Mills, E.N.C., Tatton, M.J., D'Ursel, C.C.M., Lambert, N., & Morgan, M.R.A. (1994) *J. Agric. Food Chem.* **42**, 834–840
- (59) Plumb, G.W., Lambert, N., Mills, E.N.C., Tatton, M.J., & Ursel, C.C.M. (1995) *J. Sci. Food Agric.* **67**, 511–520
- (60) Demonte, A., Carlos, I.Z., Lourenco, E.J., & Dutra de Oliveira, J.E. (1997) *Plant Foods Hum. Nutr.* **50**, 63–69
- (61) Huang, L., Mills, E.N., Carter, J.M., & Morgan, M.R. (1998) *Biochim. Biophys. Acta* **1388**, 215–226
- (62) Chidro, F.G., Anon, M.C., & Fossati, C.A. (1995) *Food Agric. Immunol.* **7**, 333–343
- (63) Koppelman, S.J., van Koningsveld, G.A., Knulst, A.C., Gruppen, H., Pigman, I.G., & de Jongh, H.H. (2002) *J. Agric. Food Chem.* **50**, 1562–1568
- (64) Griffiths, N.M., Billington, M.J., Crimes, A.A., & Hitchcock, C.H.S. (1984) *J. Sci. Food Agric.* **35**, 1255–1260
- (65) Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–254
- (66) Foucard, T., Edberg, U., & Malmheden Yman, I. (1997) *Lakartidningen* **94**, 2635–2638