Targeting the middle region of CP4-EPSPS protein for its traceability in highly processed soy-related products

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Abstract Transgenic components in genetically modified organisms consist not only of the transgenic genes, but also the transgenic protein. However, compared with transgenic DNA, less attention has been paid to the detection of expressed protein, especially those degraded from genetically modified soybean after food processing. In this study, the full length 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4-EPSPS, 47.6 kD) protein was probed with the SC-16 (S19–R33) and the DC-16 (D219–K233) polyclonal antibodies in immunoblots. Both antibodies were able to detect the full length CP4-EPSPS and its residues in soy powder made from Roundup-Ready soybeans after heating and microwaving treatments which also reduced the molecular weight of the protein to 45.8 and 38.7 kD,

respectively. Taken together the immunoblot results suggest that the middle region of the CP4-EPSPS protein possessed better stability than its N-terminal during thermal processing. This deduction was further validated by autoclave treatment, where a 37.4 kD residue of the protein was recognized by DC-16. A similar result was obtained in processed smoked sausage containing Roundup Ready soybean protein isolate (as an extender). The additional use of a further polyclonal antibody CK-17 (C372–K388), showed that compared with only the one signal for CP4-EPSPS detected by the SC-16 and CK-17 antibodies, the DC-16 middle region antibody detected four signals for CP4-EPSPS from five market sourced soy protein concentrates. Taken together, the study suggested that the middle region of CP4-EPSPS was more useful than the N- and C-terminal for tracing transgenic CP4-EPSPS protein and its remnants in highly processed soy-related products.

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Introduction

During the last two decades, there has been an increasing trend for the commercial use and the worldwide proliferation of genetically modified (GM) plants such as Roundup Ready (RR) soybean (Chen and Lin 2013). There has been some disquiet about the issue of the safety of GM plants that has been aroused from not only researchers but also consumers fanned by anti-GM activists. Globally, RR soybean contributed 75% of total world soybean production in 2011 (Bøhn et al. 2014). More countries, e.g. China, Korea, Japan, and the countries within EU, etc., have enacted legislation that demands compulsory labels of genetically modified organism (GMO) ingredients in GMO-related materials and its commercial products. The mandatory labeling threshold for GMO ingredients is defined as 0.9% in EU (Ballari and Martin 2013), 3% in Korea (Notification No. 2003-31 2000), and 5% in Japan (Notification No. 1775 2000). The concerns about GM technology include alteration in nutritional quality of foods, potential toxicity, possible antibiotic resistance from GM crops, and potential allergenicity and carcinogenicity from consuming GM foods (Uzogara 2000). However, the difficulties in strictly controlling the commercial use of GMOs, not only hinder the promotion of GMO-related products from industry to market and consumers, but also challenge the confidence between the scientific community and the public. For example, a survey conducted in Brazil revealed that 28.3% of sampled food (68 in 240) showed evidence of containing GM soy, but were not labeled explicitly as GM products (Branquinho et al. 2010).

Nowadays, most investigations of transgenic soybean, such as RR soybean, use PCR protocols for the detection of transgenic product. PCR-based methods are rapid, cheap, and quantitative (Bogani et al. 2009; Holst-Jensen et al. 2012). Most studies have been focused on DNA fragmentation and the traceability of transgenic DNA fragments (Chen et al. 2005; Onori et al. 2013). However, transgenic components from GMO's consist not only of the transgenic genes, but also the protein expressed from these genes. Although soybean proteins are widely used in many foods as functional and nutritional ingredients (Tsumura et al. 2005), less attention has been given to studying the transgenic proteins in soy-related products.

The transgenically expressed protein in glyphosate tolerant RR soybean is 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4-EPSPS) protein. This protein is expressed from a gene isolated from *Agrobacterium* sp. strain CP4

(Nida et al. 1996). The gene codes for the full length 55.6 kD CP4-EPSPS protein which consists of 527 amino acids, including a 72 amino acid chloroplast transit peptide (Gasser et al. 1988) which is cleaved off to produce a mature 47.6 kD CP4-EPSPS protein. Previously, the presence of CP4-EPSPS protein in GM soybean has been studied by using the immune-blot technique (Rogan et al. 1999; van Duijn et al. 1999; Ahmed 2002; Wu et al. 2012; Xiao et al. 2012). Not surprisingly, in food processing and production the phenomenon of CP4-EPSPS protein degradation (CP4-EPSPS protein residues detected) was found in commercial soy products (Xiao et al. 2012) and a 5% RR soybean powder processed by different thermal treatments (Wu et al. 2012).

It is well known that temperature plays an essential role in DNA and protein degradation both in non-transgenic and transgenic soybean meal under dry heating, wet heating, and extrusion processing treatments (Tian et al. 2014). However, the mechanisms underlying CP4-EPSPS degradation during food processing have yet to be elucidated. This impedes the traceability of CP4-EPSPS protein components in highly processed soy-food products.

Screening for the presence of degraded components of transgenic CP4-EPSPS protein in soy-related food stuffs will assist the inspection, quarantine and enforcement of legislation regarding products containing RR soybean. However, our knowledge about the degradation patterns of CP4-EPSPS during food processing are rare. The effort for the efficient traceability of transgenic CP4-EPSPS protein and especially its residues in highly processed soy-related products can be better achieved if the degradation patterns of CP4-EPSPS during food processing can be exploited.

The present investigation focuses on the relatively stable mid-protein region of CP4-EPSPS during food processing to enable the tracing of residual CP4-EPSPS components in highly processed soy-food products. To perform this study, three polyclonal antibodies which could specifically recognize the N-terminal (S19-R33), mid-protein region (D219-K233), and C-terminal (C372-K388) regions of the CP4-EPSPS protein, were synthesized (validated) and used. These polyclonal antibodies were named SC-16 (N-terminal), DC-16 (middle region), and CK-17 (C-terminal) respectively (Figure S1). The traceability of the CP4-EPSPS protein by using the antibodies described above was investigated in RR soybean powder after application of different thermal processing treatment. The presence of CP4-EPSPS protein residues in highly processed soy-related products was observed by immune-blot technique. Overall, our study identified the mid-protein fragment of CP4-EPSPS as the optimal target because of its traceability in highly processed soy-food products.

Materials and methods

Materials

Milled soybean powders of RR soybeans (RR, GM, event 40-3-2) and non-transgenic soybeans (WT) were kindly provided by Jiangsu Province Entry-Exit Inspection and Quarantine Bureau, China. Five soybean protein concentrates were also purchased separately from different free markets in China: Anyang (Henan Province), Nantong (Jiangsu Province), Hangzhou (Zhejiang Province), Nanjing (Jiangsu Province), and Anheng (Anhui Province).

Heat processing treatment

One gram of the powder of RR and WT soybean was placed in different 1.5 mL Eppendorf tubes and placed into a water bath (DK-BD, Shanghai Yiheng Technology Co., Ltd., Shanghai, China). Heat processing operations (95 °C) in the water bath were carried out at the following time periods: 5, 15, 30, 60, and 120 min. All the processing steps were carried out in triplicate. Special attention was paid to avoid cross-contamination between the RRS and WT soybean powders.

Microwave treatment

Three grams of soybean powder of RR and WT were uniformly spread across a Petri dish (85 mm) and heated in a microwave oven (MWO; WP700P21, Galanz, Shunde, China) at 750 W for the following time periods: 1, 5, 10, 20, and 30 min. RR and WT soybean powder were of course spread in different petri dishes. The MWO exposure was restricted to 30 min since higher exposure of microwave resulted in charring of the milled flour. All the processing steps were carried out in triplicate. Special attention was paid to avoid cross-contamination between RRS and WT soybean powder.

Autoclave treatment

Three grams of soybean powder of RR and WT were packed in a gauze bag and transferred to a 1 L Schott bottle for autoclaving. RR and WT soybean powder were stored in different bottles. An automated high-pressure steam autoclave (LDZX-30KBS, Shanghai Shenan Medical Instrument Factory, Shanghai, China) was used, and set at 121 °C and 0.1 MPa pressure for the following time periods: 10, 20, 30, 60, and 120 min. The indicated time did not include the time required for raising and lowering of the temperature in the autoclave. All the processing steps were carried out in

triplicate. Special attention was paid to avoid the cross-contamination between RR and WT soybean powder.

Preparation of smoked RR-sausage with the addition of 8% RR soybean isolated proteins

Briefly, pork dorsal muscles were sourced, to which were added 2–3% w/w NaCl, 0.025–0.05% w/w NaNO₂, and 0.03–0.05% w/w ascorbate. To this meat, 8% RR protein isolates (produced by Mantianxue Ltd. Henan, China) were added and then mixed by chopping with 30% ice for ~15 min. The mixtures were then filled into sausage casing under vacuum. Finally, raw sausages were processed by baking for 1 h at 85 °C, followed by steaming for 1 h at 75 °C, and then by another 30 min of smoking at 50 °C. Samples were collected at the following stages, baking, steaming, and smoking.

Protein extraction

A protein extract was prepared from 0.1 g soybean powder, 0.1 g free market sourced soybean protein concentrates (please refer to the “Materials” section for location details), and 0.1 g processed smoked RR-sausages (grounded into powder by using liquid nitrogen). Proteins were also extracted from 0.3 g fine powder of 2-week-old seedlings of commercial alfalfa (*Medicago sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), cucumber (*Cucumis sativus*), and rice (*Oryza sativa*). All samples were extracted in 1 mL cold extraction buffer (150 mM NaCl, 8 mM Na₂HPO₄·12H₂O, 2 mM KH₂PO₄·H₂O, 4 mM KCl, 0.05% w/v Tween-20, and 2% W/V SDS, pH7.4). After shaking for 30 min at room temperature, the mixture was centrifuged twice at 20,000 g (model Avanti J-25, Beckman) for 15 min. Total protein concentration in the supernatant was then determined by BCA (Bicinchoninic acid) assay (Multisciences Biotechnology Co. Ltd., Hangzhou, China), using bovine serum albumin (BSA) as the standard.

Preparation of polyclonal antibodies

In our experiment, three peptide sequences of 15–17 amino acid residues each corresponding to the specific amino acids regions of the 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4-EPSPS) (Genbank: AAL67577.1) sequence, were obtained by chemical synthesis (GL Biochem (Shanghai) Ltd.). and within the N-terminal (S19GTVRIPGDK-SISHR33), the middle region (D219ADGVRTIRLE GRGK233), and C-terminal (C372DEGETSLVVRG RPDGK388) of the protein (Figure S1), and were used as the antigens. The sequences were hydrophilic, surface-oriented,

and flexible. The synthetic peptides were purified by HPLC and coupled to keyhole limpet haemocyanin (KLH) carrier. Then, the purified and KLH coupled three peptides were used to produce specific rabbit (male New Zealand white rabbit) polyclonal antibodies. The ELISA titre for these three polyclonal antibodies was $> 16,000$. All the above mentioned works for the preparation of polyclonal antibodies and determination of titer were carried out by GL Biochem (Shanghai) Ltd.

Immuno-blotting analysis

The method of immune-blotting analysis is conventional. Briefly, 30 μ g of protein extracted from soybean powder after the various thermal treatments, smoked RR soybean sausage, and different plant materials (described previously), were subjected to SDS-PAGE using a 12.5% w/v acrylamide resolving gel (Mini Protean II System, Bio-Rad, Hertz, UK). The separated protein was then transferred to polyvinylidene difluoride (PVDF) membranes, and non-specific binding of antibodies was blocked with 5% w/v non-fat dried milk in phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. Membranes were then probed with primary antibodies diluted 1:3,000 in PBS buffer containing 1% w/v non-fat dried milk. Antigen-antibody protein complexes were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (1:3000 dilution) (Takara, Dalian, China). The color was developed with a solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) as HRP substrate. Prestained protein molecular marker (Fermentas) and protein molecular weight marker (Takara) were used. In addition, an identical SDS-PAGE gel was stained by Coomassie Brilliant Blue R-250 to confirm equal loading of the protein samples.

Results and discussion

Polyclonal antibody specificity of DC-16

The specificity of the DC-16 polyclonal antibody towards the middle region (D219–K233) of the CP4-EPSPS protein (Figure S1) was examined by using recombinant His-tagged CP4-EPSPS protein. The *Cp4-epsps* gene (GenBank: AB209952) was cloned and a recombinant His-tagged CP4-EPSPS protein (66.4 kD), consisting of mature CP4-EPSPS protein (47.6 kD), 6 $\times$ His-tag (0.8 kD), and the translated vector sequence (18.0 kD), was collected. Briefly, genomic DNA from RR soybean was extracted by the modified CTAB method (Xiao et al. 2012). Primers (F: 5'-CGCGAATTCATGGCACAAATTAACAACAT-3', R: 5'-GCTCTCGAGTCAGGCAGCCTT-CGTATC-3') were

designed by using Primer Premier 5.0 to clone the *Cp4-epsps* gene. The PCR product was gel-purified and cloned into pMD-19 T vector (Takara, Dalian, China) for sequencing (Genscript, Nanjing, China). Then, the mature *Cp4-epsps* gene without the predicted chloroplast transit peptide was subcloned into the *E. coli* expression vector pET-32a (Novagen, Madison, US) to produce *pET-32a-Cp4-epsps*. The construct integrity was verified by both the restriction analysis and complete DNA sequencing of the insert (Genscript, Nanjing, China). According to the manufactures' instructions (Novagen, Madison, U.S.), the recombinant His-tagged CP4-EPSPS protein was induced by 0.1 mM IPTG (isopropyl β -D-thiogalactopyranoside) at 28 °C for 3 h in *E. coli* Rosseta (OD₆₀₀ = 0.6) (Figure S2). Then the recombinant His-tagged CP4-EPSPS protein was purified through Ni-NTA column (Genscript Biotechnology Co. Ltd., Nanjing, China) and Sephadex G-25 chromatography (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Only a single band of ~ 67.0 kD appeared in PVDF membrane by using DC-16 polyclonal antibody against the recombinant His-tagged CP4-EPSPS protein (Figure S3), demonstrating that the DC-16 polyclonal antibody can identify the CP4-EPSPS protein. The specificity of the DC-16 antibody was further demonstrated by applying it in immunoblots of WT soybean powder, alfalfa, wheat, barley, cucumber, and rice plant extracts as negative controls (Figure S3). As can be expected with polyclonal antibodies (Charleston et al. 2001; Ahmed 2002; Wu et al. 2012; Xiao et al. 2012), some minor non-specific bands were also observed on the PVDF membrane. We not only tested the specificity of the antibodies used, but also subjected the wild-type soybean powder as a negative control to clarify the identity of non-specific bands. In part, an explanation for the existence of non-specific bands may be that the EPSPS family is ubiquitous in plants and microorganisms (Ueda et al. 1993). The specificity of the other polyclonal antibodies, SC-16 polyclonal antibody (N-terminal region, S19-R33) and CK-17 polyclonal antibody (C-terminal region, C372-K388), has previously been substantiated by Xiao et al. (2012).

Detection of the 45.8 kD residual fragment of CP4-EPSPS protein in RR soybean powder after heat treatment

During processing for soy related food products, proteins are typically subjected to harsh conditions to achieve desired food quality outcomes—for example thermal processing, changes in pH, reducing agents, mechanical shearing etc. These processing procedures drastically change the physical characteristics of the proteins, often leading to denaturation and loss of protein function (Hammond and Jez 2011). Figure 1a shows that the full length CP4-EPSPS protein band

(47.6 kD) was detected by the SC-16 polyclonal antibody in RR soybean powder after heat treatment up to 120 min. However, by using the DC-16 polyclonal antibody, both the full length protein and a 45.8 kD residue band were observed after heat treatment for 15 min (Fig. 1c). It is proposed that the mature CP4-EPSPS protein is partially degraded and reduced in size by the thermal processing treatment. In addition, it is observed that the stability of the middle region of CP4-EPSPS protein was better than its N-terminal region under heat, suggesting that the thermo-stability of N-terminal of CP4-EPSPS protein is worse than its middle region. Actually, the relative molecular mass of the region from N-terminal of CP4-EPSPS to the antigen region recognized by the SC-16 polyclonal antibody (MILHGASSRPA-TARKSSGL18) is approximately 1.8 kD (calculated by ExPASy, http://web.expasy.org/compute_pi/). This is in accordance with an approximately 45.8 kD residue band detected by the DC-16 rather than SC-16 polyclonal antibody. No immune signal was detected in protein extracts of heat treated WT soybean powder (Fig. 1b, d). However, no obvious changes of total protein pattern from either the RR or WT soybean powder were observed after heat treatment for 120 min (Fig. 1e, f).

Detection of the 38.7 kD residual fragment of CP4-EPSPS protein in RR soybean powder after microwave treatment

Microwave treatment is another commonly used thermal food processing treatment that may impact the CP4-EPSPS protein from RR soybean (van Remmen et al. 1996). In comparison with the lack of obvious changes in total protein levels in RR and WT soybean after heat treatment (Fig. 1e, f), there was a remarkable degree of degradation of total protein observed after microwave treatment of RR (after 20 min treatment) and WT soybean powder (at 30 min treatment) (Fig. 2e, f).

The full length CP4-EPSPS protein was observed in RR soybean powder after microwave treatment up to 30 min, but no residual protein bands were detected by the SC-16 polyclonal antibody (Fig. 2a). By contrast, a 38.7 kD residue band was detected by the DC-16 polyclonal antibody in the RR soybean powder after 20 min of treatment (Fig. 2c). It was therefore suggested that the antigen region probed by SC-16 polyclonal antibody partially degraded into small peptides during microwave treatment. These results suggest that the middle region of CP4-EPSPS protein exhibited greater stability than its N-terminal region under microwave processing, and the N-terminal of CP4-EPSPS protein might exposed and degraded firstly during microwave processing. No immune-detection signals were observed in WT soybean powder under microwave treatment (Fig. 2b, d).

Detection of the 23.4 and 37.4 kD residues of CP4-EPSPS protein in RR soybean powder after autoclave treatment

Autoclaving simulates moist heating under pressure when applied to manufacture soy drinks, textured vegetable protein, soybean meal, maize instant tortilla, etc. (Vijayakumar et al. 2009). As depicted from the SDS-PAGE results, no significant change of the total protein content was observed at 10 min of autoclave treatment (Fig. 3e, f). These observations were in accordance with the previous studies that showed that there were similar levels of crude protein after autoclave treatment (127 °C, 10 min), as in non-autoclaved soybean meal samples (Sadeghi et al. 2006). After 10 min of autoclave treatment, however, charring was observed on the treated samples. This is consistent with the reduction of total protein in both RR and WT soybean with autoclave treatment (Fig. 3e, f), and especially observation of only faint protein bands after 120 min of autoclave treatment.

The severity of degradation of total protein was more evident in the WT soybean than transgenic RR soybean powder. This indicated that transgenic RR soybean may have a higher resistance to autoclaving than that of WT soybean powder. Regardless, no full length of mature CP4-EPSPS protein was detected after 20 min treatment by using the SC-16 polyclonal antibody, except for the existence of a very faint residue band at 23.4 kD (except 120 min; Fig. 3a). In contrast, the full length CP4-EPSPS protein was detected by the DC-16 polyclonal antibody at all time points, along with a 37.4 kD residue band that appeared after 20 min of autoclave treatment (Fig. 3c). These results also suggest that the middle region of CP4-EPSPS protein showed better stability than the N-terminal region with respect to autoclave treatment. Again, it suggested that N-terminal of CP4-EPSPS protein might exposed and then degraded firstly during processing. It has been showed that the roasting process significantly reduced the percentage of α -helixes and increased the percentage of β -sheets in the golden flax seeds (Yu 2005). Actually, by using the online protein tertiary structure prediction tool SWISS-MODEL (<https://swissmodel.expasy.org/interactive/eYtcnW/models/>), it showed that part of the antigen motif recognized by the SC-16 (N-terminal) is mainly α -helix, and the secondary structure of the whole antigen motif for the DC-16 (middle region) belongs to β -sheets and β -turns. This might be one possible explanation for the higher efficiency of DC-16 to detect the full length of CP4-EPSPS protein in thermal processed RR soybean related products than that of SC-16 polyclonal antibody. Similar to the results in the above sections, no immune-detection signals were detected in WT soybean powder with the same treatment procedures when using either SC-16 or DC-

Fig. 1 Immune-blot analysis of the degradation of CP4-EPSPS protein in heat processed GM-soy (GM) and non-GM-soy (WT). Lane M, Protein molecular weight marker (**a–d**, prestained protein molecular marker; **e** and **f**, protein molecular weight marker); Lane GM, unprocessed GM-soy; samples processed by heat treatment for 5, 15, 30, 60, and 120 min were loaded into corresponded lanes; Lane WT, unprocessed non-GM-soy. The antibodies used were the SC-16

(**a** and **b**) and DC-16 (**c** and **d**), respectively. Corresponding Coomassie brilliant blue-stained gels of **a–d** are also provided (**e** and **f**). The *solid black* and *purple arrow* show the immune-reactive bands corresponding to full length and the degradation fragment of CP4-EPSPS protein (47.6 and 45.8 kD), respectively. The *dashed black arrows* presented in all immune-blot were due to non-specific staining (color figure online)

16 polyclonal antibodies (Fig. 3b, d). Not surprisingly, the total protein detected with Coomassie brilliant stained SDS-PAGE, showed more rapid and severe degradation with autoclave treatment than the simple heating and microwave treatments (Figs. 1e, f, 2e, f, and 3e, f).

Given the complexity of the protein degradation process, CP4-EPSPS protein might occur simultaneously with different products/residues after harsh treatments such as autoclaving. The full length protein, modified full length protein, partly degraded protein with degraded residues, and even some small polypeptides, could be assumed to be present. After 20 min of autoclave treatment, for example, the full length CP4-EPSPS protein in the treated RR soybean samples was still detected by the DC-16 polyclonal antibody; however, this full length protein could not be detected by the SC-16 antibody. Presumably autoclaving modified CP4-EPSPS protein such that there was a loss of immunochemical recognition. This concurs with our earlier observation of the instability of the N-terminal with respect to thermal treatment, which was especially apparent with harsher thermal treatment (i.e. autoclaving), and also resulted in loss of antigenicity detectable by SC 16 polyclonal antibody. Therefore, thermal modification of the protein (Smales et al. 2002) and/or the loss of

immunochemical recognition or the failure of immune detection as a consequence of the complex mechanisms involved in food processing (Ahmed 2002; Ueda et al. 1993; Brett et al. 1999; de Luis et al. 2009), are most likely explanations for the absence of detection of full length CP4-EPSPS by SC-16 polyclonal antibody. Furthermore, our results suggest that the CP4-EPSPS protein might be more thermostable than other endogenous soy-proteins in the treated RR and WT soybean and samples during thermal processing.

A further explanation of the above observations might be the possibility that the content of the exposed full length CP4-EPSPS protein with non-impaired antigenicity is too low to be detected by SC-16 antibody after 20 min autoclaving. Indeed, previous studies reported nearly a two-fold reduction in CP4-EPSPS protein detected in transgenic soybean meal that was extruded at 120 °C, compared with 90 °C (Tian et al. 2014). Faint immune-detection signals for the full length CP4-EPSPS protein were still evident with the SC-16 polyclonal antibody after 10 min autoclaving (Fig. 3a). A further explanation could be that the solubility of the residues produced upon autoclaving might be greater in comparison to the full length CP4-EPSPS. This deduction has been confirmed previously, as it has

Fig. 2 Immune-blot analysis of the degradation of CP4-EPSPS protein in microwave processed GM soy (GM) and non-GM soy (WT). Lane M, protein molecular weight marker (a–d, prestained protein molecular marker; e and f, protein molecular weight marker); Lane GM, unprocessed GM- soy; samples processed by microwave for 1, 5, 10, 20, and 30 min were loaded into corresponded lanes; Lane WT, unprocessed non-GM soy. The antibodies used were the SC-16

(a and b) and DC-16 (c and d), respectively. Corresponding Coomassie brilliant blue-stained gels of a–d are also provided (e and f). The *solid black* and *purple arrow* show the immune-reactive bands corresponding to full length and the degradation fragment of CP4-EPSPS protein (47.6 and 38.7 kD), respectively. The *dashed black arrows* presented in all immune-blot were due to non-specific staining (color figure online)

been found that the solubility of soybean protein is increased after heat treatment, although the subunit of soy protein was degraded (Kinsella 1979).

Taken together, 45.8 (heating), 38.7 (microwaving), and 37.4 kD (autoclaving) residues were probed by using the DC-16 antibody, along with the detection of full length CP4-EPSPS (Figure S4a). However, no residue but only the full length CP4-EPSPS protein was probed by using the SC-16 antibody under heating and microwaving treatment. The DC-16 antibody showed better ability to detect CP4-EPSPS residues during thermal processing than the SC-16 antibody (Figure S4a–d).

Traceability of CP4-EPSPS in processed smoked sausages with the addition of RRS isolated proteins, and in market-sourced soybean protein concentrates

Smoked sausages with the addition of RR soybean isolated proteins (8%) (RR soybean-sausage) were used to test the traceability of CP4-EPSPS by using the polyclonal antibodies mentioned above. As expected, after baking, steaming, and smoking processes, no band was detected by SC-16 antibody in processed smoked RRS-sausages (Fig. 4a), whereas a clear band was simultaneously detected by DC-16 (Fig. 4b). This is in agreement with the

instability of the N-terminal of CP4-EPSPS with respect to autoclaving (Fig. 3). Further, DC-16 and the CK-17 polyclonal antibody (C-terminal, C372–K388), were applied to consider the traceability of CP4-EPSPS in market-sourced soybean protein concentrates. Both CK-17 (Fig. 5b) and SC-16 (Wu et al. 2012) only detected the signal of CP4-EPSPS in one of five different market-sourced soybean protein concentrates. In contrast, four of five the samples were detected with the DC-16 antibody (Fig. 5a). Aside from the full length CP4-EPSPS, residues of CP4-EPSPS protein were detected by DC-16 but not SC-16 and CK-17 antibodies. This suggests that DC-16 antibody possesses better ability to detect CP4-EPSPS and its residues in highly processed soya-related products than other two antibodies.

Conclusion

Taken together, our results showed that the DC-16 (middle region, D219–K233) polyclonal antibody probed more CP4-EPSPS and its residues in thermally processed RR soybean powder, smoked RRS sausages and also market-sourced soybean protein concentrates than SC-16 (N-terminal, S19–R33) and CK-17 (C-terminal, C372–K388)

Fig. 3 Immune-blot analysis of the degradation of CP4-EPSPS protein in autoclave processed GM soy (GM) and non-GM soy (WT). Lane M, protein molecular weight marker (a–b, prestained protein molecular marker; e and f, protein molecular weight marker); Lane GM, unprocessed GM soy; samples processed by autoclave treatment for 10, 20, 30, 60, and 120 min were loaded into corresponded lanes; Lane WT, unprocessed non-GM soy. The

antibodies used were the SC-16 (a and b) and DC-16 (c and d). Corresponding Coomassie brilliant blue-stained gels of a–d are also provided (e and f). The solid black, blue and purple arrows show the immune-reactive bands corresponding to the full length (47.6 kD), and two degradation fragments of CP4-EPSPS protein (23.4 and 37.4 kD). The dashed black arrows presented in all immuno-blot were due to non-specific staining (color figure online)

Fig. 4 Immune-blot analysis of the traceability of CP4-EPSPS protein in smoked sausage with the addition of 8% RR soybean isolated proteins (smoked RR soybean sausage). Lane M, protein molecular weight marker (a, b, prestained protein molecular marker); Lane GM, unprocessed GM soy; RRS-sausage with baking, steaming, and smoking processing were loaded into corresponded lanes; Lane

IP, RRS isolated proteins. The antibodies used were SC-16 (a) and DC-16 (b), respectively. The solid black, arrows show the immune-reactive bands corresponding to full length CP4-EPSPS protein (47.6 kD). The dashed black arrows presented in all immuno-blot were due to the non-specific staining

antibody. These results suggest that targeting the middle region, rather than N- and C- terminal, of CP4-EPSPS protein, might be an optimal strategy for tracing CP4-EPSPS and especially its residues in highly processed soy-products (Figure S5). Thus, not surprisingly, the DC-16 antibody was exploited and applied to the colloidal gold strip technique (detection limits, 1%; Huang et al. 2012) for monitoring and detecting the CP4-EPSPS and its residues in highly processed soy-related products. Furthermore, by

using mass spectrometry techniques e.g. ESI-Q-ToF or MALDI ToF/MS, the middle region of CP4-EPSPS protein might be a potential target for investigating whether CP4-EPSPS degradation during thermal processing is random or in a specific manner (alpha helices, beta sheets, and/or beta turns dependent or independent etc.).

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Fig. 5 Immune-blot analysis of the traceability of CP4-EPSPS protein in market-sourced soybean protein concentrates. Lane M, protein molecular weight marker (**a**, and **b**, prestained protein molecular marker); Lane GM, unprocessed GM-soy; market-sourced samples from Anyang (Henan province), Nantong (Jiangsu province), Hangzhou (Zhejiang province), Nanjing (Jiangsu province), and Anhang (Anhui province) were loaded into corresponded lanes; Lane WT, unprocessed non-GM-soy. The antibodies used were the DC-16 (**a**) and CK-17 (**b**), respectively. The *solid black arrows* show the immune-reactive bands corresponding to the full length, and three degradation fragments of CP4-EPSPS protein (47.6, 46.5, 41.9, and 35.5 kD). The *dashed black arrows* presented in all immune-blot were due to non-specific staining

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References

- Ahmed FE (2002) Detection of genetically modified organisms in foods. *Trend Biotechnol* 20:215–223
- Ballari RV, Martin A (2013) Assessment of DNA degradation induced by thermal and UV radiation processing: implications for quantification of genetically modified organisms. *Food Chem* 141:2130–2136
- Bogani P, Minunni M, Spiriti MM, Zavaglia M, Tombelli S, Buiatti M, Mascini M (2009) Transgenes monitoring in an industrial soybean processing chain by DNA-based conventional approaches and biosensors. *Food Chem* 113:658–664
- Bøhn T, Cuhra M, Traavik T, Sanden M, Fagan J, Primicerio R (2014) Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. *Food Chem* 153:207–215
- Branquinho MR, Ferreira RTB, Cardarelli-Leite P (2010) Survey of compliance with labeling legislation in food containing GMOs in Brazil. *J Food Compos Anal* 23:220–225
- Brett GM, Chambers SJL, Huang L, Morgan MRA (1999) Design and development of immunoassays for detection of proteins. *Food Control* 10:401–406
- Charleston B, Fray MD, Baigent S, Carr BV, Morrison WI (2001) Establishment of persistent infection with non-cytopathic bovine viral diarrhoea virus in cattle is associated with a failure to induce type I interferon. *J Gen Virol* 82:1893–1897
- Chen H, Lin YG (2013) Promise and issues of genetically modified crops. *Curr Opin Plant Biol* 16:255–260
- Chen Y, Wang Y, Ge Y, Xu B (2005) Degradation of endogenous and exogenous genes of Roundup-Ready soybean during food processing. *J Agric Food Chem* 53:10239–10243
- de Luis R, Lavilla M, Sánchez L, Calvo M, Pérez MD (2009) Immunochemical detection of Cry1A(b) protein in model processed foods made with transgenic maize. *Eur Food Res Technol* 229:15–19
- Gasser CS, Winter JA, Hironaka CM, Shah DM (1988) Structure, expression, and evolution of the 5-enolpyruvylshikimate-3-phosphate synthase genes of petunia and tomato. *J Biol Chem* 263:4280–4287
- Hammond BG, Jez JM (2011) Impact of food processing on the safety assessment for proteins introduced into biotechnology-derived soybean and corn crops. *Food Chem Toxicol* 49:711–721
- Holst-Jensen A, Bertheau Y, de Loose M, Grohmann L, Hamels S, Hougs L, Morisset D, Pecoraro S, Pla M, Van den Bulcke M, Wulff D (2012) Detecting un-authorized genetically modified organisms (GMOs) and derived materials. *Biotechnol Adv* 30:1318–1335
- Huang M, Shen W, Wu H, Xiao X, Xu S, Zhou X, He J, Zhou G (2012) Polyclonal antibody performing specific antigen-antibody reaction with CP4-EPSPS protein and application thereof. Patent, CN ZL 201010225353.3
- Kinsella JE (1979) Functional properties of soy protein. *J Am Oil Chem Soc* 56:242–258
- Nida DL, Kolacz KH, Buehler RE, Randy Deaton W, Schler WR, Armstrong TA, Taylor ML, Ebert CC, Rogan GJ, Padgett SR, Fuchs RL (1996) Glyphosate-tolerant cotton: genetic characterization and protein expression. *J Agric Food Chem* 44:1960–1966
- Onori R, Lopardo R, de Giacomo M, de Santis B, Prantera E, Palmaccio E, Brera C (2013) Traceability of genetically modified Roundup Ready soybean: a case study on sampling and analytical uncertainty along processing chain. *Food Control* 34:494–501
- Notification No. 2003-31 (2000) Ministry of Agriculture and Forestry of Korea, Seoul, Korea, April 22, 2000
- Notification No. 1775 (2000) Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, Tokyo, Japan, June 10, 2000
- Rogan GJ, Dubin YA, Lee TC, Magin KM, Astwood JD, Bhakta NS, Leach JN, Sanders PR, Fuchs RL (1999) Immunodiagnostic methods for detection of 5-enolpyruvylshikimate-3-phosphate synthase in Roundup Ready[®] soybeans. *Food Control* 10:407–414
- Sadeghi AA, Nikkha A, Shawrang P, Shahrehabak MM (2006) Protein degradation kinetics of untreated and treated soybean meal using SDS-PAGE. *Anim Feed Sci Technol* 12:122–131
- Smales MC, Pepper DS, James DC (2002) Protein modification during anti-viral heat-treatment bioprocessing of factor VIII concentrates, factor IX concentrates and model proteins in the presence of sucrose. *Biotechnol Bioeng* 77:37–48
- Tian F, Guan Q, Wang X, Teng D, Wang J (2014) Influence of different processing treatments on the detectability of nucleic acid and protein targets in transgenic soybean meal. *Appl Biochem Biotechnol* 172:3686–3700

- Tsumura K, Saito T, Tsuge K, Ashida H, Kugimiya W, Inouye K (2005) Functional properties of soy protein hydrolysates obtained by selective proteolysis. *LWT-Food Sci Technol* 38:255–261
- Ueda K, Fukushima H, Masliah E, Xia Y, Iwai A, Yoshimoto M, Otero DAC, Kondo J, Ihara Y, Saitoh T (1993) Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proc Natl Acad Sci USA* 90:11282–11286
- Uzogara SG (2000) The impact of genetic modification of human foods in the 21st century: a review. *Biotechnol Adv* 18:179–206
- van Duijn G, van Biert R, Bleeker-Marcelis H, Peppelman H, Hessing M (1999) Detection methods for genetically modified crops. *Food Control* 10:375–378
- van Remmen HHJ, Ponne CT, Nijhuis HH, Bartels PV, Kerkhof PJAM (1996) Microwave heating distributions in slabs, spheres and cylinders with relation to food processing. *J Food Sci* 61:1105–1114
- Vijayakumar KR, Martin A, Gowda LR, Prakash V (2009) Detection of genetically modified soya and maize: impact of heat processing. *Food Chem* 117:514–521
- Wu HH, Zhang Y, Zhu CQ, Xiao X, Zhou XH, Xu S, Shen WB, Huang M (2012) Presence of CP4-EPSPS component in Roundup Ready soybean-derived food products. *Int J Mol Sci* 13:1919–1932
- Xiao X, Wu HH, Zhou XH, Xu S, He J, Shen WB, Zhou GH, Huang M (2012) The combination of quantitative PCR and western blot detecting CP4-EPSPS component in Roundup Ready Soy plant tissues and commercial Soy-related foodstuffs. *J Food Sci* 77:C603–C608
- Yu P (2005) Protein secondary structures (α -helix and β -sheet) at a cellular level and protein fractions in relation to rumen degradation behaviours of protein: a new approach. *Br J Nutr* 94:655–665