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Sensitization Potency of Sunflower Seed Protein in a Mouse Model: Identification of 2S-Albumins More Allergenic Than SFA-8

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Scope: Food allergy to sunflower seed (SFS) protein is not frequent and only non-specific lipid transfert protein (nsLTP) Hel a 3 is officially recognized as a food allergen. Out of the eleven seed storage 2S-albumins (SESA) detected in SFS, only SFA-8 allergenicity has been investigated so far. The study aimed then to evaluate SFS protein allergenicity and particularly, to compare the sensitization potency of SESA in a mouse model.

Methods and Results: The most abundant SESA and nsLTP were isolated from SFS through a combination of chromatographic methods. Purified proteins were then used to measure specific IgG1 and IgE responses in BALB/c mice orally sensitized to different SFS protein isolates. The study, thus, confirmed the allergenicity of SFA-8 and Hel a 3 but mice were also highly sensitized to other SESA such as SESA2-1 or SESA20-2. Furthermore, competitive inhibition of IgE-binding revealed that SFA-8 IgE-reactivity was due to cross-reactivity with other SESA. 11S-globulins were weakly immunogenic and were rapidly degraded in an in vitro model of gastroduodenal digestion. In contrast, Hel a 3, SESA2-1 and SFA-8 were more resistant to proteolysis and gastroduodenal digestion did not affect their IgE-reactivity.

Conclusions: SESA2-1 or SESA20-2 were more potent allergens than SFA-8 in this mouse model. Allergenicity of SESA must be now confirmed in SFS-allergic patients.

1. Introduction

Sunflower seeds (*Helianthus annuus*, SFS) have been consumed as early as 3000–5000 BC by native Americans as a source of flour for bread.[1] Nowadays, sunflower is one of the most widely cultivated plant for oil production. The residual oil cake is also used for livestock feeding because of its high content in protein. Introduction of SFS protein into human nutrition appears as a good candidate to diversify protein sources for sustainable human consumption.

Use of SFS proteins as food ingredient requires the production of affordable protein isolates of good nutritional quality. In this regard, SFS proteins exhibit a relatively well-balanced composition in amino acids with a slightly limiting content in lysine and a high content in methionine and cysteine.[2] The seed contains two main types of storage proteins: the 11S-globulins (or helianthinins) and the seed storage 2S-albumins (SESA).

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The SESA are water-soluble and can represent 20% to 60% of the total storage protein.[3,4] At the protein level, 11 different SESA have been identified in SFS but SESA2, SFA-8 (or SESA3) and SESA20-2 are predominant.[5] In contrast to the single monomeric SFA-8, some SESA such as SESA2 encode heterodimeric preproalbumins leading after maturation to SESA2-1 and SESA2-2. SFA-8 also differs from the other SESA by an unusual amount of 16 methionine residues in addition to the eight cysteine residues involved in the four conserved disulfide bridges characteristic of the 2S-albumins.[4,6] Because of its good emulsification properties, SFA-8 has been previously studied for food industry application.[7–9] In contrast, the other SESA have been rarely investigated because of the difficulty to obtain sufficient amount of single homogeneous components.[5,7]

Although SFS are consumed all over the world and have not been assigned for mandatory allergen labeling in the European Union, the use of SFS protein isolates as food ingredient requires allergenic risk assessment. In this regard, seeds are generally considered as a source of food allergens with a risk for anaphylaxis. Therefore, sensitization to SFS has been investigated in the EuroPrevall project because of its frequent consumption in some countries and its potential to cause food allergy reactions.[10] That study reported a prevalence of sensitization to SFS up to 5% of adults in European countries while seroprevalence reached 8.7% in India.[10,11] Fortunately, subjects sensitized to SFS have been described as tolerant, i.e., not developing clinical symptoms after ingestion, in a high proportion of cases.[12] Nevertheless, several cases of anaphylactic reaction after ingestion of SFS have been reported.[13–16] Crespo et al. reported that 56% of Spanish patients allergic to vegetables other than legumes were allergic to SFS.[17] In Athens, SFS belonged to the foods most commonly causing probable food allergy, which was defined as the combination of self-reported food allergy and matching IgE-sensitization.[18]

So far, allergic reactions to SFS have been attributed to two proteins: the non-specific lipid transfer protein (nsLTP) Hel a 3[16,19] and SFA-8.[20] Although SFA-8 has been reported as an IgE-binding protein, it has been also considered as a weak allergen in comparison with other 2S-albumins such as Ber e 1 from Brazil nut.[21] To our knowledge, no investigation about the allergenic potency of other SESA has been reported. Cross-reactivity between SFS proteins and other food allergens is also expected. For example, Comstock et al. reported a possible co-sensitization to SFS in peanut-allergic patients[22] and Ukleja-Sokolowska et al. reported a partial IgE cross-reactivity between Hel a 3 and the peanut nsLTP Ara h 9.[19]

In the present work, we aimed to evaluate de novo sensitization to SFS protein isolates differentially enriched in SESA/nsLTP and 11S-globulins and to compare in particular that to the different SESA. For this purpose, we produced homogeneous fractions of the most abundant 2S-albumins and we developed a mouse model of oral sensitization to SFS proteins. The potential IgE cross-reactivity among SESA and also between SFS and peanut allergens was further investigated with sera from mice sensitized to SFS and peanut proteins. Finally, we evaluated the impact of in vitro gastroduodenal digestion on the IgE-immunoreactivity of SFS proteins.

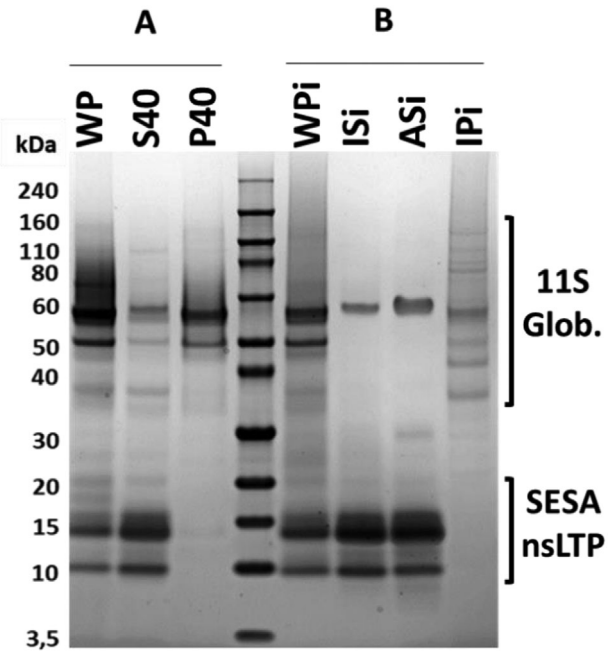

Figure 1. SDS-PAGE analysis of SFS protein extracts. A) Protein extracts generated for protein purification: a SFS whole protein extract (WP) was produced in-house and the corresponding fractions obtained after precipitation with 40% ammonium sulfate. The soluble fraction S40 contained mostly SESA and nsLTP while the insoluble fraction P40 contained mostly the 11S-globulins. B) SFS protein isolates used for mice sensitization: WPi corresponds to a whole protein isolate, ISi to the soluble protein fraction obtained after isoelectric precipitation of WPi, ASi to the soluble protein fraction obtained after acidification of the cold press meal and IPi to the insoluble protein fraction obtained after isoelectric precipitation of WPi (see Supporting Information). Analysis performed under non-reducing conditions (5 µg protein/lane).

2. Results

2.1. Fractionation of SFS Protein

Protein from the whole SFS protein extract (WP) was first separated by 40% ammonium sulfate precipitation. The 2S-albumins and nsLTPs, with molecular weight (MW) lower than 20 kDa, were mostly recovered in the soluble fraction (S40) while the 11S-globulin acidic and basic subunits, with MW higher than 30 kDa, were mostly found in the precipitated fraction (P40, **Figure 1**). Additional 2D electrophoresis showed that 2S-albumins and nsLTP presented rather homogeneous physicochemical pI. In contrast, 11S-globulins displayed a wider range of pI varying between 5 and 9 (Figure S1, Supporting Information). The S40 fraction was further used to purify 2S-albumins and nsLTP in order to develop immunoassays (see below).

Moreover, four different isolates were used to orally sensitize mice to SFS protein (see Section 4). The whole protein isolate (WPi) displayed an electrophoretic protein pattern similar to the WP extract produced in-house. Two isolates, ISi and ASi, were enriched in 2S-albumins while IPi was enriched in 11S-globulin subunits (Figure 1).

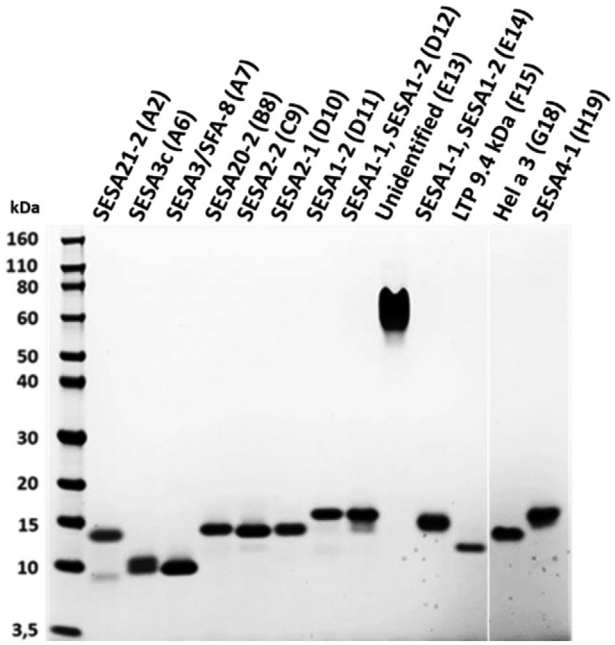

Figure 2. SDS-PAGE analysis of purified SFS fractions obtained after a combination of IEC and RP-HPLC. Corresponding fraction identification number are indicated and their identification by MS analysis is presented in Table S1, Supporting Information. Analysis was performed under non-reducing conditions (0.5 µg protein/lane).

2.2. 2S-albumins Purification and Identification

Proteins from S40 were first fractionated by ion exchange chromatography (IEC) and then further separated by reversed-phase high performance liquid chromatography (RP-HPLC) (see Supporting Information). Finally, 13 well-resolved fractions were obtained and corresponded to several SFS proteins purified near to homogeneity for most of them (**Figure 2**). Protein identification was performed by mass spectrometry (MS) analysis of purified protein and corresponding tryptic peptides (Table S1). Although SFS 2S-albumins share close physicochemical properties, we succeeded to isolate the most abundant ones: SESA1-2, SESA2-1, SESA2-2, SFA-8 and SESA20-2 (Figure 2, fractions D11, D10, C9, A7 and B8, respectively). An additional isoform of SFA-8, SESA3c, was also co-purified with SFA-8 (Figure 2, fraction A6). Of note, SESA2-1 was purified as a mix of two isoforms that differ by one threonine residue at the C-terminus, as previously reported.[6] SESA21-2 and SESA4-1 were purified with lower MW than expected and they probably corresponded to naturally post-translationally truncated forms. Finally, in addition to Hel a 3, another nsLTP (Uniprot assessing number: A0A251SK36, fraction F15, Figure 2) was also isolated but the nsLTP SDI-9 (Q39950), which was previously described by Berecz et al., was not detected.[7] Only the proteins exhibiting the expected MW, i.e., SESA2-1, SESA20-2, SFA-8 and Hel a 3 (Figure 2, fractions D10, B8, A7 and G18, respectively) were used to develop immunoassays for the characterization of humoral and cellular responses in in vivo studies.

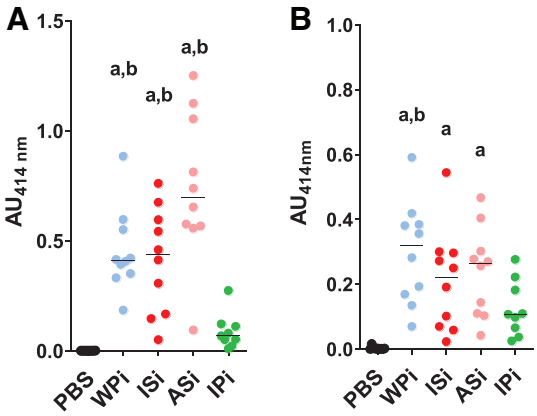

Figure 3. Specific IgG1 A) and IgE B) responses to a SFS whole protein (WP) in mice orally sensitized to different SFS protein isolates. Results are reported in absorbance units (AU) at 414 nm. Data were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparison post-test. Median is indicated. "a," significantly different from PBS group; "b," significantly different from IPi group.

2.3. De Novo Sensitization to SFS Protein

Oral sensitization of mice to each of the four isolates induced the development of specific humoral responses (**Figure 3**). However, mice sensitized to WPi, ISi and ASi developed higher levels of IgG1 and IgE specific to SFS proteins than mice sensitized to IPi (Figure 3). The highest levels of sensitization correlated then with the presence of 2S-albumins and nsLTP in the isolates. Indeed, mice that received WPi, ISi and ASi were highly sensitized to SESA2-1, SESA20-2, SFA-8 and, to a smaller extent, Hel a 3 while most of the mice that received IPi did not develop significant IgG1 and IgE responses against 2S-albumins and nsLTP (**Figure 4**). We also investigated the sensitization against some purified fractions of 11S globulin subunits but specific IgG1 levels were about 50 to 100-fold lower than those measured against SESA, with no significant differences between mice groups (data not shown).

At the end of the protocol, ex vivo splenocyte reactivation with purified SESA2-1, SESA20-2, SFA-8 and Hel a 3 was performed to assess cellular responses and specific cytokines secretion. In line with the humoral responses, sensitization to SFS proteins was confirmed by strong secretions of Th2 cytokines IL-4, IL-5 and IL-13. Low levels of IL-10 and IFN-γ were also detected (**Figure 5**). Among the different groups of mice, the highest levels of Th2 cytokines secretion were observed in mice that received ASi.

2.4. IgE Cross-reactivity Between SESA

Considering the structure homology between SESA (Table S2 and Figure S2, Supporting Information), we aimed then to determine the level of IgE cross-reactivity between the different 2S-albumins. For this purpose, we performed competitive inhibition of IgE-binding to SESA20-2, SESA2-1 and SFA-8. As shown in **Figure 6**, a high level of cross-reactivity was evidenced between SESA. According to a sequence identity of 92% between SESA2-2 and SESA20-2, these two SESA displayed similar

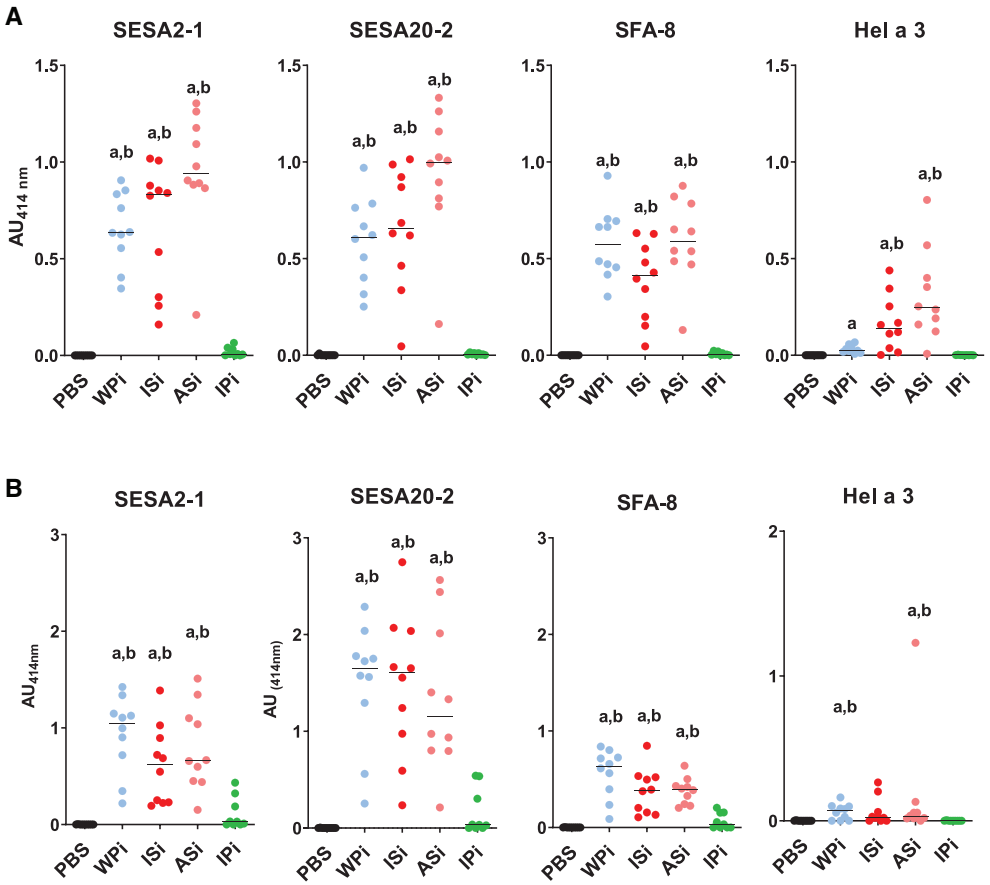

Figure 4. Specific IgG1 A) and IgE B) responses to SESA2-1, SESA20-2, SFA-8 and Hel a 3, in mice orally sensitized to different SFS protein isolates. Results are reported in absorbance units (AU) at 414 nm. Data were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparison post-test. Median is indicated. "a," significantly different from PBS group; "b," significantly different from IPi group.

inhibitory capacities (Figure 6A). Nevertheless, an IgE cross-reactivity of about 50% was also observed between SESA2-1 and SESA20-2, which share a sequence identity of only 31% (Figure 6A,B). More surprisingly, SFA-8 exhibited the lower inhibitory capacity, even for inhibiting IgE-binding to it-self (Figure 6C) thus indicating that IgE-binding to SFA-8 was not due to IgE antibodies primarily specific to SFA-8 but rather to cross-reactivity with IgE antibodies specific to other SESA. These results, including the low inhibitory capacity of SFA-8, were also replicated in mice that were orally sensitized to the WP extract produced in-house (Figure S3, Supporting Information).

We investigated then the potential cross-reactivity between SFS and peanut protein. When testing sera from SFS-sensitized mice, no significant IgG1 and IgE responses against peanut proteins were detected (Figure S4A, Supporting Information). Similarly, no significant IgG1 and IgE responses against SFS proteins were observed in sera from peanut-sensitized mice (Figure S4B, Supporting Information).

2.5. In Vitro Gastroduodenal Digestion

We finally studied the impact of in vitro gastroduodenal digestion on the IgE reactivity of SFS protein. First, the IPi isolate exhibited a low resistance to proteolytic digestion, as evidenced by partial or total disappearance of high MW proteins and the persistence of small peptides around 5 kDa after 60 min of gastric digestion. These peptides disappeared then rapidly after 1 min of duodenal digestion (**Figure 7**A). Conversely, ASi (and ISi, Figure S5) exhibited a very strong resistance to gastroduodenal digestion with a very limited impact of gastric digestion and the persistence of large resistant peptides (>10 kDa) after duodenal digestion (Figure 7B). This was further confirmed by the gastroduodenal digestion of purified Hel a 3, SFA-8 and SESA2-1. Hel a 3 appeared to be resistant to gastroduodenal digestion and even to a pepsin resistance test performed with an enzyme-to-substrate ratio of 3:1 (Figures 7C and S6, Supporting Information). For SFA-8, only a shift to slightly higher MW could be observed when performing SDS-PAGE analysis under non-reducing conditions. However, under reducing conditions, a peptide at around 5 kDa appeared during gastric digestion while two resistant peptides (at 4 and 6 kDa) could be observed throughout duodenal digestion of SFA-8 (Figure 7D). Contrary to SFA-8, SESA2-1 was highly resistant to gastric digestion. Even when using a higher pepsin-to-substrate ratio, we did not observe a significant degradation of SESA2-1 while SFA-8 was no more detected after 15 min of digestion (Figure S6, Supporting information). For SESA2-1, only the duodenal digestion led to

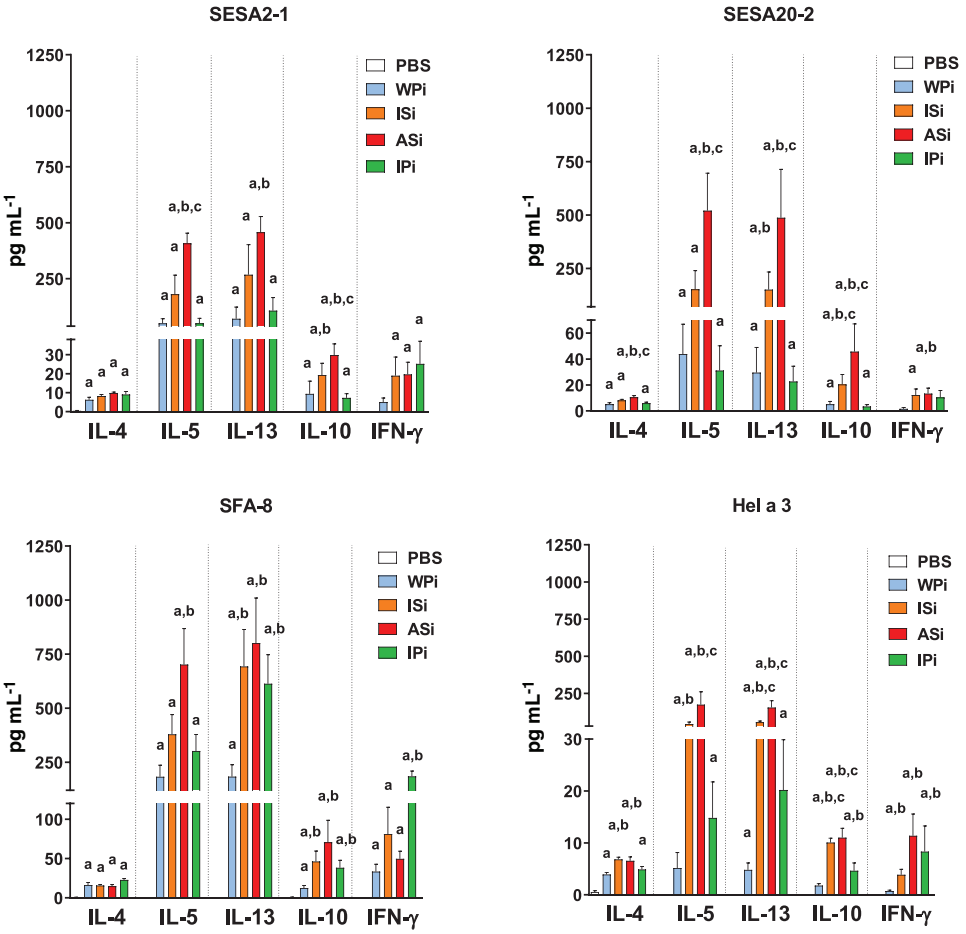

Figure 5. Cytokines secretion by splenocytes reactivated with SESA2-1 A), SESA20-2 B), SFA-8 C) and Hel a 3 D). Mice were orally sensitized to different SFS protein isolates: WPi, ISi, ASi or IPi. Results are represented as cytokines secretion (pg mL^{-1}) in supernatant of reactivated splenocytes after subtraction of cytokines assayed in supernatants of PBS-reactivated splenocytes thus corresponding to specific production. Data were analyzed using one-way ANOVA and Tukey multiple comparison test. "a," significantly different from PBS group; "b," significantly different from WPi group and "c," significantly different from IPi group.

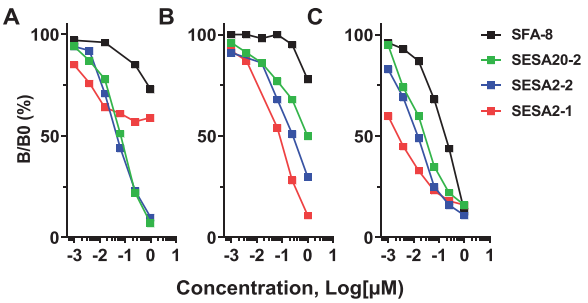

Figure 6. IgE cross-reactivity between different SESA as evaluated by competitive inhibition of IgE-binding to SESA20-2 A), SESA2-1 B) and SFA-8 C). Competitive assay was performed with a pool of sera from mice sensitized to the isolates containing SESA, i.e., WPi, ISi and ASi (see Section 4). These results were replicated with mice sensitized to the WP extract (Figure S3, Supporting Information).

the production of two fragments of around 4 kDa and 8 kDa (Figure 7E). However, despite the detection of breakdown products during the gastroduodenal digestion, the IgE-immunoreactivity of both SFA-8 and SESA2-1 was not affected as revealed by the unaltered capacity of the digested proteins to inhibit IgE-binding (**Figure 8**).

3. Discussion

Introduction of novel food ingredients into human nutrition requires to improve the strategy of allergy risk assessment. Therefore, there is a continued interest in the development of suitable animal models that could provide a more holistic approach, in particular for evaluating de novo sensitization.[23] Such models would be even more useful when history of exposure is unknown or when rare cases of allergy toward homolog allergens would suggest an allergenic potential of the assessed proteins. So far, SFS food allergens have been rarely investigated and only one allergen, Hel a 3, is actually considered as a food allergen by the WHO/IUIS.[24] It is still unclear whether SFA-8 is a food allergen although it has been shown to be an IgE-binding protein. We, thus, aimed to compare in a mouse model the level of sensitization toward SFS proteins including SFA-8 and the other

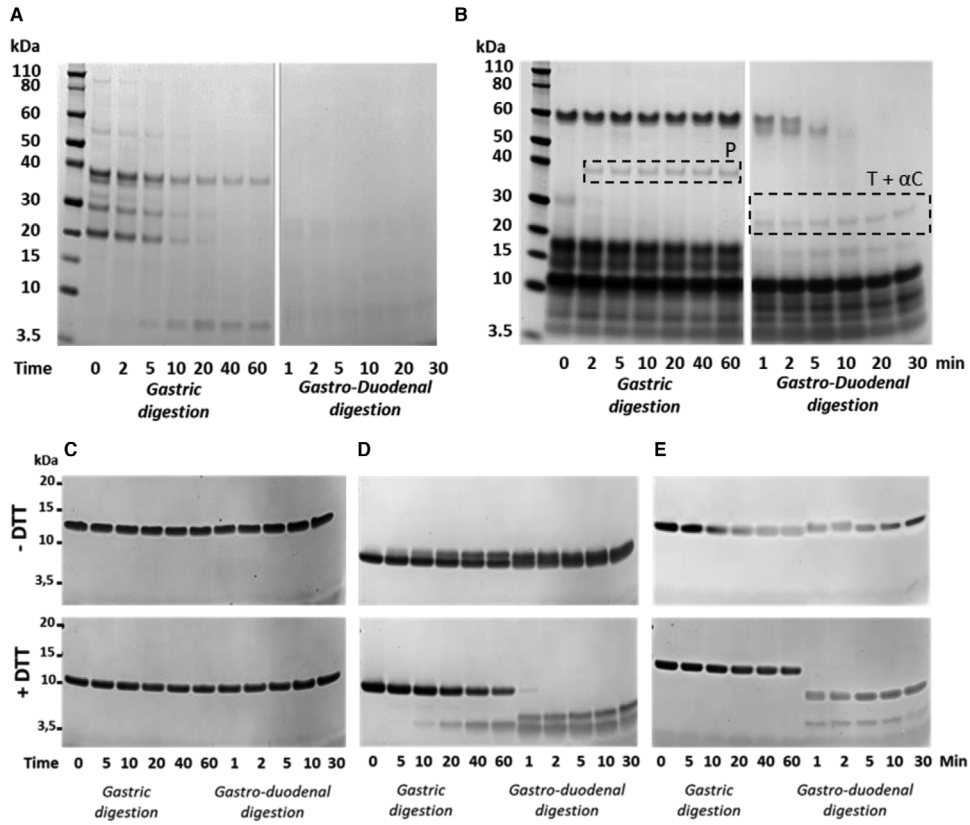

Figure 7. SDS-PAGE analysis of gastroduodenal digestion of SFS proteins. Digestion of IPi A), ASi B) or ISi (Figure S5, Supporting Information) were analyzed under reducing conditions (10 µg protein/lane). Digestion of Hel a 3 C), SFA-8 D) and SESA2-1 E) were analyzed under non-reducing (- DTT) and reducing (+ DTT) conditions (1 µg protein/lane). Time of sample collection is indicated. P, pepsin; T, trypsin; αC, α-chymotrypsin.

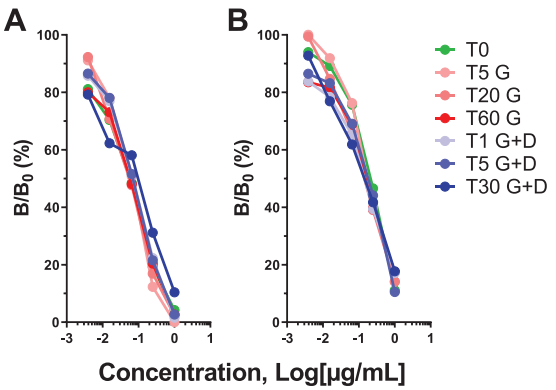

Figure 8. IgE-immunoreactivity of digested SESA2-1 A) or SFA-8 B). Competitive inhibition of IgE-binding to SESA2-1 or SFA-8 by the corresponding digested protein after 0, 5, 20 and 60 min of gastric digestion (G) and after 1, 5, and 30 min of gastroduodenal digestion (G+D, see Section 4). The in vitro digestion of SESA2-1 and SFA-8 did not affect their IgE-immunoreactivity.

SESA. The mouse model was also intended to provide sera for the evaluation of the residual IgE-reactivity of digested SFS proteins.

First, we purified the most abundant SESA. Previous characterization of the different SESA was impaired by the difficulty to produce homogeneous preparations of each 2S-albumin. Jayasena et al. purified minute amount of SESA to perform MS analysis,[5] while Berecz et al. characterized three mixtures differentially enriched in SESA.[7] Here, several 2S-albumins were purified near to homogeneity so that the most abundant and the most homogeneous fractions could be used to develop immunoassays.

Oral administrations of different SFS isolates to mice then emphasized de novo sensitization to the SESA. Indeed, the lowest levels of IgG1 and IgE to SFS proteins were observed in mice sensitized to IPi mostly composed of 11S-globulins. In contrast, the highest levels of specific IgG1 and IgE, and of cytokines secretion, were detected in mice sensitized to the extracts containing the highest concentrations of SESA and Hel a 3, i.e., ASi and ISi. We then confirmed the IgE-immunoreactivity of SFA-8. However, it also appeared that other 2S-albumins such as SESA2-1 and SESA 20–2 displayed a higher allergenic potency. This is in agreement with Kelly et al. who showed by immunoblotting assay that IgE from SFS-allergic patients recognized not only SFA-8 but also several other proteins with a higher MW, ranging from 12 kDa to 21 kDa.[20] Moreover, competitive inhibition of IgE-binding revealed that the IgE-reactivity of SFA-8 was mostly due to the binding of cross-reactive IgE antibodies primarily specific to other SESA. In this regard, sensitization to SFA-8 resulted probably from structure similarities with other SESA. Interestingly, Kean et al. also suggested that, compared to the Brazil nut 2S-albumin, SFA-8 was a weak

allergen because it could promote a Th1 response and/or reduce the Th2 bias of the T cell response.[21] Here, it can be noted that splenocyte reactivation by SFS proteins induced IFN-γ secretion, as previously described in experimental models using cholera toxin as adjuvant.[23,25] In line with the study of Kean et al.,[21] the highest levels of IFN-γ secretion were observed in splenocytes reactivated with SFA-8 (Figure 5). However, underlying cellular mechanisms that could involve intrinsic immunomodulatory properties of SFA-8, remain to be elucidated.

Potential Ig cross-reactivity between SFS and peanut proteins was also investigated and no significant IgG1- and IgE-reactivity to peanut proteins was detected in SFS-sensitized mice. This absence of cross-reactivity was partly expected when considering the low sequence identity between SESA and peanut 2S-albumins Ara h 2 and Ara h 6 (Table S2, Supporting Information). However, in contrast to the abundant peanut storage proteins Ara h 1, Ara h 2, Ara h 3 and Ara h 6, concentration of Ara h 9 in the whole peanut protein extract was probably too low to detect a potential cross-reactivity between Ara h 9 and Hel a 3, which are 48% identical. This particular cross-reactivity should be then investigated in experimental models focusing on LTP allergenicity, for example, by sensitizing mice to high amount of purified LTP as previously described by Rodriguez et al.[26] Cross-reactivity between Hel a 3 and Ara h 9 would be even more adequately investigated in patients co-sensitized to several nsLTP, as observed in the Mediterranean area.[27]

Finally, de novo sensitization to SFS proteins could be only partially related to their resistance to proteolysis. Indeed, 11S-globulins subunits were quickly degraded during gastroduodenal digestion and accordingly, mice sensitized to IPi developed significantly lower levels of specific IgG1 and IgE antibodies. In contrast, SESA were highly resistant to proteolytic degradation and were the most allergenic SFS proteins. Nevertheless, among SESA, we showed that sensitization occurred primarily against SESA20-2 or SESA2-1 rather than to SFA-8. This difference of potency between 2S-albumins could be due to the lower resistance to gastroduodenal digestion of SFA-8 compared to SESA2-1, as observed in the present study or as previously described by Berecz et al.[28] This difference of stability toward digestion was further evidenced when performing a pepsin resistance test. However, in more physiologic conditions, the gastroduodenal digestion did not affect the IgE immunoreactivity of SFA-8 and SESA2-1, as shown by competitive inhibition of IgE-binding performed with sera from sensitized mice. Furthermore, Hel a 3 was totally resistant to digestion but remained less allergenic than the SESA. Considering that Hel a 3 and SFA-8 are the two most abundant proteins in the soluble fraction of SFS protein, resistance to proteolytic digestion could be then related only to the low level of sensitization to SFA-8, but not to Hel a 3, compared to SESA2-1 or SESA20-2.

To conclude, we observed in our mouse model of oral sensitization to SFS that IgE sensitization was primarily directed against SFS 2S-albumins such as SESA2-1 or SESA20-2 and that IgE-binding to SFA-8 was mostly due to IgE-cross-reactivity. In this regard, SFS isolates enriched in 2S-albumins may then present a higher allergenic risk for human consumption than the isolate enriched in 11S-globulins. However, these differences of sensitization potency among SESA should be now confirmed in patients allergic to SFS proteins and the panel of SFS proteins purified in this study will allow such investigation. The present study also illustrates the difficulty to assess the allergenic risk of a food protein since SFA-8 appeared as a weak allergen despite being an IgE-binding protein while other SESA seemed to present a higher risk of sensitization.

4. Experimental Section

SFS Protein Extraction: SFS cold press meal obtained after oil extraction (OLEAD, Pessac, France) was ground with an Ultra-Turrax homogenizer and defatted (see Supporting Information). After resuspension of the defatted sample in 20 volumes of extraction buffer (w/v, 100 mM sodium borate pH 9.0, 500 mM NaCl, 0.9% n-Octyl-β-D-glucopyranoside) for 4 h at RT and centrifugation at 3000 x *g* for 30 min at 4 °C, the supernatant containing soluble proteins was collected and the pellet was resuspended in 10 volumes of 4 M urea w/v for 2 h at RT and centrifuged for extraction of insoluble proteins. The two collected supernatants were separately dialyzed against 20 mM sodium phosphate buffer (pH 7.4, 3.5 kDa cut-off) and then pooled. The resulting whole SFS protein extract (WP) was stored at −20 °C.

Protein Fractionation, Purification, and Biotinylation: Proteins contained in the WP extract were first fractionated using 40% ammonium sulfate precipitation (see Supporting Information). After centrifugation (8000 x *g*, 30 min, 4 °C), the supernatant (S40) and the precipitate (P40, resuspended in 50 mM potassium phosphate, 0.5 M NaCl, pH 7.4) were dialyzed separately against 20 mM sodium phosphate pH 7.4 buffer (3.5 kDa cut-off). Protein fractionation from S40 was carried on by performing IEC followed by RP-HPLC (see Supporting Information). Final fractions were freeze-dried before characterization.

Biotinylation of purified SFS protein was performed with EZ-link NHS-PEG4-biotin according to manufacturer's instructions (Thermo Fisher Scientific). The protein:biotin molar ratio was at least 1:5 and was adjusted by taking into account the number of primary amines in each biotinylated protein. The level of labeling was followed by MS analysis and biotinylation was carried on until unlabeled protein was no more detected. Biotinylation was stopped by addition of 1 M Tris buffer.

Protein Characterization: SDS-PAGE analysis was performed with precast gel (NuPage 4–12% Bis-Tris gels). Purified proteins were characterized by MS using an Ultraflex MALDI TOF/TOF apparatus (Bruker Daltonics, GmbH). Proteomic analysis was performed following in-solution digestion procedures (Pierce Trypsin Protease, MS Grade, Thermo Fisher Scientific). Mass spectra were analyzed by using Peptide Analysis software (Bruker Daltonics, GmbH) and protein identification was carried on by using Peptide Mass (ExPASy server)[29] and a custom-built database.

SFS Protein Isolates: SFS isolates were provided by LRGP (Nancy, France) and produced from the cold press meal (OLEAD, Pessac, France) as previously described with minor modifications.[30] Protein was extracted as described in Section 4.1. The whole protein isolate (WPi) corresponded to proteins soluble in 0.3 M NaCl solution at pH 7.3 after a combination of microfiltration and ultrafiltration processes (see Supporting Information). Isoelectric precipitation of WPi protein was then performed at pH 3.5. The resulting insoluble fraction corresponded to the IPi isolate while the ISi isolate contained the soluble proteins. Another isolate, ASi, contained soluble proteins extracted after direct acidification (pH 3.0) of the cold press meal resuspended with water (see Supporting Information).

Sensitization of Mice to SFS Protein Isolates: Female BALB/cByJ mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed under standard breeding conditions and were acclimated for 2 weeks. All experiments were performed according to the European Community rules on animal care and the study design was approved by the local ethics committee CETEA (Ethical approval number: A16-079). Mice received a standard diet (LASQCdiet Rod16-H, Genobios, Laval, France) free of SFS, peanut or soybean protein. Seven-week-old mice (n = 10/group) were orally sensitized to the different protein isolates, i.e., WPi, ISi, ASi or IPi, on day 1, 8, 15, 22, 29, and 36 by intragastric administrations (300 µL/mouse) of 5 mg

of protein mixed with 10 µg of cholera toxin (Sigma-Aldrich, St Louis, USA). A control group (n = 10) received only cholera toxin in saline buffer (PBS). Blood samples were collected on day 43 from the retro-orbital venous plexus on mice anesthetized with isoflurane (Belamont, Nicholas Piramal Limited, London, UK). After blood collection, mice were euthanized by cervical dislocation to collect spleens for ex vivo reactivation. In order to evaluate IgE cross-reactivity between SFS and peanut proteins, another group of mice (n = 10) were orally sensitized to a whole protein extract from roasted peanut following the same protocol of intragastric administrations as described above.[31]

Humoral Responses: Specific IgG1 levels to SFS protein were measured using an enzyme allergosorbent test with proteins (2.5 µg mL^{-1}) passively adsorbed on microtiter plates.[32] After ON incubation at 4 °C with diluted sera (1:200,000), IgG1-binding was revealed by the addition of acetylcholinesterase (AChE)-labeled anti-mouse IgG1 monoclonal antibody. Ellman's reagent was then used to determine AChE activity and absorbance was measured at 414 nm.

In order to avoid the masking of coated allergens by specific IgG, specific IgE levels were determined by using a reverse enzyme allergosorbent test that measures the binding of biotinylated allergens by serum IgE antibodies captured by a specific monoclonal antibody immobilized on the solid phase. Briefly, rat anti-mouse IgE monoclonal antibody (Clone LOME-3, AbD Serotec, Biorad) was adsorbed on microtiter plates (2.5 µg mL^{-1}). After ON incubation with diluted sera (1:25, 50 µL/well), plates were washed and biotinylated SFS proteins were added (0.05 $nmol.mL^{-1}$, 50 µL/well) for 4 h at RT. After washing, AChE-labeled neutravidin was added before revelation with Ellman's reagent.

For competitive inhibition of IgE-binding, a pool of sera from mice sensitized to WPi, ISi and ASi was used. After ON incubation with 50 µL/well of diluted serum (1:25), 25 µL of inhibitors (i.e., increasing concentration of SFS protein) were mixed with 25 µL of biotinylated SFS protein, and incubated at RT for 4 h. IgE-binding was then revealed as described above. Results were expressed as B/B0, B0 and B representing the amount of labeled SFS protein bound to immobilize IgE antibodies in the absence or presence of a known concentration of inhibitor, respectively.

Splenocyte Reactivation and Cytokines Secretion: Spleens were pooled by pairs (5 pools $group^{-1}$) and were mechanically homogenized (GentlMACS Dissociator, Miltenyi Biotec, USA). After lysis of red blood cells with lysing buffer (Red Blood Cell Lysing Buffer Hybrid-Max, Sigma-Aldrich), splenocytes were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin and 100 mg mL^{-1} streptomycin. Cells (10^6 cells $well^{-1}$) were incubated for 60 h at 37 °C (5% CO_2) in 96-well culture plates in the presence of purified SFS protein (20 µg mL^{-1}) or PBS. Polymyxin B sulfate salt (50 µg mL^{-1}, Sigma-Aldrich) was added to each activator for endotoxin neutralization. Cytokines secretion (IL-4, IL-5, IL-10, IL-13, and INF-γ) was measured in duplicate in collected supernatants by using Bioplex 200 System and commercial multiplexed kits according to the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France).

In Vitro Gastroduodenal Digestion: According to Mandalari et al., SFS extracts (10 mg) or purified proteins (1 mg) were resuspended into simulated gastric fluid (0.15 M NaCl, pH 2.5).[33] Samples were incubated at 37 °C during 10 min and gastric digestion was started by adding pepsin (182 U mg^{-1}). Aliquots (200 µL) were collected at 0, 2, 5, 10, 20, 40 and 60 min. Gastric digestion was stopped by adding 0.5 M ammonium bicarbonate at pH 8. Duodenal digestion was then performed by adjusting pH to 6.5 with simulated duodenal fluid (0.15 M NaCl, pH 6.5). A simulated bile salt solution buffer (4 mM taurocholate sodium, 4 mM sodium glycodeoxycholate, 25 mM Bis-Tris buffer, 0.44 U mg^{-1} α-chymotrypsin, and 34 U mg^{-1} trypsin, pH 6.5) was then added at 37 °C. Aliquots (200 µL) were collected at 1, 2, 5, 10, 20 and 30 min. Reactions were stopped by adding protease inhibitor cocktail (Sigma-Aldrich).

Statistical Analysis: Data on humoral responses were analyzed using nonparametric Kruskal-Wallis test with Dunn's multiple comparison test. Analysis of cytokines secretion by splenocytes was performed on log transformed data and using one-way ANOVA with Tukey multiple comparison test. Statistical analyses were performed with GraphPad Prism 8.01 software and $p < 0.05$ was considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

SH and HB share senior authorship. JA, MG, BG, HB and SH performed the experiments. HB, KAP and SH designed the study. RK provided SFS isolates. OG supervised the PRODIAL project. HB, KAP, OG and SH supervised the study. JA, HB and SH wrote the manuscript. All authors approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

2S-albumin, allergen, cross-reactivity, IgE, nsLTP, seed, sunflower

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