

The BASALIT multicentre trial: Gly m 4 quantification for consistency control of challenge meal batches and towards Gly m 4 threshold data

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Abstract

Scope: The BASALIT clinical trial (EudraCT 2009-011737-27) investigated efficacy of birch allergen immunotherapy on lowest observed adverse effect levels (LOAEL) after soy food challenge in patients with birch-associated and Gly m 4 allergen-mediated soy allergy. Thus, consistently stable Gly m 4 levels were required in standardised challenge meals.

Methods and results: Soy meal included soy protein isolate (SPI, 88% total protein). A Gly m 4-specific ELISA was developed and validated. 6 SPI and 24 meal batches were analysed for Gly m 4. (Repeated-measures) analyses of variance were done to identify potential changes between batches/ in time. Gly m 4 was below the ELISA detection limit (2 ng/ml) in placebo batches. With < 20 % mean coefficient of variation, Gly m 4 levels were consistent in 24 soy meal batches and within individual 12 weeks shelf-life.

Conclusion: The novel Gly m 4-specific ELISA proved consistency of challenge meal batches over a 56 months study period. With an average of 178 µg/g Gly m 4 in SPI, Gly m 4-LOAEL can be calculated once clinical LOAEL data based on SPI are available. Hence, sensitivity of patients can be correlated to the relevant allergen content instead of total protein of the allergenic source.

1 Introduction

Soybean is considered an important allergenic food and requires labelling in the European Union when used as an ingredient in compound foods [1]. Allergy to soybean and products thereof may be elicited by both allergenic storage proteins, such as Gly m 5 and Gly m 6 [2], and by Gly m 4, which is the soy allergen component that is homologous to the major birch pollen allergen Bet v 1 [3, 4]. While Gly m 5 and Gly m 6 appear to be relevant allergens in primary sensitisation to soybean, e.g. in children [2, 5], in birch pollen-associated soy allergy, Gly m 4 is the major allergen [3–5], and severe allergic reactions, including anaphylaxis, have been reported [3, 6]. Interestingly, the amount of Gly m 4 in soybean is low, with between 8 and 638 µg/g quantified in various soybean lines [4, 7–9]. The double blind placebo controlled food challenge (DBPCFC) is considered as gold standard in the diagnosis of food allergy [10, 11]. In the BASALIT multi-center clinical trial (EudraCT 2009-011737-27) the effect of sub-cutaneous allergen immunotherapy with recombinant Bet v 1 on pollen related food allergy to soybean mediated by Gly m 4 was investigated. In order to identify potential changes of individual patient sensitivity with regard to minimal eliciting doses of soy, titrated soy DBPCFC was performed before and after Bet v 1-specific immunotherapy using a cocoa-based active soy food challenge meal and a corresponding soy-free placebo meal (Treudler et al., under revision). Titrated DBPCFC with increasing cumulative amounts of food allergen allows determining lowest observed adverse effect levels (LOAEL) for individual and population specific allergen risk assessment [12, 13]. Usually, sensitivity is determined in relation to the total protein amount ingested [13]. However, in birch pollen-associated soy allergy mediated by Gly m 4, this approach may be inaccurate because the relevant allergen presents only a minor component compared to the total protein fraction of approximately 35 % of total soybean weight [14]. In order to provide comparable challenge meals to all clinical centers and throughout the entire BASALIT multi-center trial, standardised soy food challenge meals were produced centrally in a pharmacy, and controlled for microbiological contaminations and consistency of the Gly m 4 content (Treudler et al., under revision). Here, we describe the development, validation and application of a novel Gly m 4-specific ELISA for the verification of Gly m 4 batch-to-batch consistency of the challenge meals. Other methods for

Gly m 4-quantification in various soybean lines have been described [4, 7–9] but not validated for and applied in a complex food matrix such as the BASALIT cocoa-based food challenge meal. Provided that Gly m 4 monosensitisation predominates in birch-associated soy allergy, to the best of our knowledge, this is the first study to control consistency of each soy challenge meal batch with regard to the relevant allergen content in a complex food matrix and throughout the entire study period. In addition, accurate quantification of the Gly m 4 amounts in the challenge source material may allow determining the individual patient sensitivity related to the relevant allergen instead of total protein [15]. Knowledge about LOAEL related to individual allergen components will improve our understanding for a better management, diagnosis and immunotherapy of immediate-type allergic reactions to foods.

2 Material and methods

2.1 Generation of recombinant Gly m 4

rGly m 4 (UniProt entry P26987) was expressed as a soluble, C-terminally hexahistidine tagged protein in *E. coli* BL21. Expressed rGly m 4 was purified using IMAC on chelating Sepharose FF and ion exchange chromatography on Q Sepharose FF, followed by buffer exchange on G25Fine. Purified rGly m 4 was stored in MOPS buffer, pH 7.6 (20 mM 3-(N-morpholino-) propane sulfonic acid, 150 mM NaCl). A mean protein concentration of 4.31 ± 0.05 mg/ml was determined by photometric A_{280} absorbance measurement of three replicate 1:5 dilutions, and using an extinction coefficient of 0.43 per mg/ml, as calculated on the basis of the amino acid sequence of the expressed protein. Silver-stained SDS-PAGE showed a single band slightly below the 21.5 kDa marker and no visible Gly m 4 fragments or contaminating expression host proteins. Analytical gel filtration showed a single symmetrical peak indicating a monomeric and homogeneously folded protein (details not shown).

2.2 Food challenge meals

Food challenge meals were prepared as described elsewhere (Treudler et al., under revision. Briefly, soy challenge meal was made of nine increasing levels of soy protein isolate (SPI) (Supro®760IP, Uelzena eG, Uelzen, Germany) in a blinding matrix of cocoa, carob, oat flakes, rice flour, and sugar (table 1). In placebo meal, SPI was substituted by hypoallergenic Sinlac® soy free powder (Nestlé Nutrition GmbH, Frankfurt, Germany). 24 individual batches of active soy challenge meal and placebo meal were prepared over a period of 56 months. At the clinical trial sites for placebo-controlled soy food challenges in soybean allergic study subjects, defined amounts of tap water were added to freshly prepare the active and placebo challenge meals which were applied in a randomized order near in time on two different days. For *in vitro* analysis using ELISA, the dry powdered material was extracted and analyzed. At weeks 0, 6 and 12, each of the individual batches of active soy meals (levels 4 and 9) were analysed in triplicate extracts for Gly m 4 consistency. In addition, each of the placebo batches (level 9), and of SPI material used in the preparation of the individual active soy meals were analysed for Gly m 4 in triplicate extracts at week 0.

At weeks 0, 6 and 12, all batches of challenge meals were additionally analysed for microbiological contaminations (Institute of laboratory medicine, Clinical chemistry and molecular diagnostics, University Hospital, Leipzig, Germany). No significant microbiological impurities were detected in any batch within these 12 weeks.

2.3 Sample preparation for ELISA analysis

Prepackaged challenge meal samples were already homogenized during the production (Treudler et al., under revision). Challenge meals were provided as dry powdered materials in closed containers as it was done to the trial centres, too. Dry powdered challenge meal batches were stored under identical conditions as in the clinical centres, i.e. dry and at ambient temperature for a period of 12 weeks for each batch. Prior to ELISA analysis, a corresponding vial of an active soy challenge dose or placebo was additionally homogenized during 2 min mixing using a spatula. 500 milligram of sample material were extracted in 5 ml of Tris-Tricine (TT) sample extraction buffer (8 mM tris-hydroxymethyl aminimethane, 25 mM tricine, 2 mM Calciumlactate, pH 8.6) [16]. Extraction was performed within

1 h at ambient temperature, using an overhead shaker. After additional shaking for 1 h, extracts were centrifuged at 11,000 *g* and 4°C for 15 min. Obtained supernatant was stored at -20°C until analysis. Each of the individual batches of active soy meals (levels 4 and 9) were extracted at weeks 0, 6, and 12, and all placebo and SPI batches at week 0. From each sample, three individual extracts were prepared and analyzed using ELISA. Each sample extract was diluted (1:20, 1:50, 1:100, 1:250) in ELISA incubation buffer and each of the 4 dilutions analyzed in a single well.

2.4 ELISA reagents and standards

Buffers: Coating buffer was phosphate-buffered saline (PBS), pH 7.1, consisting of 10 mM phosphate and 150 mM sodium chloride, blocking buffer consisted of PBS and 2 % bovine serum albumin (BSA), washing buffer consisted of PBS and 0.05 % Tween 20, incubation buffer consisted of washing buffer and 0.1 % BSA, substrate buffer, pH 3.9, consisted of 210mM citric acid monohydrate and 300 mM potassium hydroxide, and substrate solution consisted of 1 mM 3,3',5,5'-tetramethylbenzidine (TMB) and 3 mM H₂O₂ in substrate buffer.

Standards and reagents: rGly m 4 ('lot 1'), as described above, was stored in aliquots at 4° C until used, and allowed analysis of 61 ELISA microplates (ELISA 'lot 1'). The activity of additional rGly m 4 ('lot 2'), kindly provided by Prof. Paul Rösch, University of Bayreuth, Germany, was adjusted to the activity of the rGly m 4 ('lot 1') by titration using ELISA, and allowed the analysis of 45 ELISA microplates (ELISA 'lot 2'). rGly m 4 standard curves contained five standards between 1.22 and 312.5 ng/ml (ELISA 'lot 1'), and seven standards between 0.96 and 700 ng/ml (ELISA 'lot 2'), each freshly prepared by fourfold and threefold serial dilution in ELISA incubation buffer, respectively.

Rabbit antiserum raised against rGly m 4 [4] was stored in aliquots at - 20°C until used.

Controls: SPI (pilot batch M350033395) and active soy challenge meal level 8 (table 1, pilot batch 20090911) served as in-house reference preparations (IHRPs) in all ELISA runs. Extracts of both IHRPs were extracted in TT sample extraction buffer as described above, and aliquots for single day use were stored at - 20°C until used. Extracts of IHRP of SPI were diluted (1:200, 1:500, 1:1000,

1:2000) in ELISA incubation buffer and analyzed in single wells per dilution. Extracts of IHRP of active soy challenge meal level 8 were diluted (1:250) in incubation buffer and analyzed in duplicate wells.

2.5 Gly m 4 specific inhibition ELISA

Certified 96-well microplates (Maxisorp, Nunc, via SigmaAldrich, Taufkirchen, Germany) were coated overnight with 50 ng/well rGly m 4 in 100 µl/well coating buffer. Wells were blocked with blocking buffer for 2 h. rGly m 4 standards, extracts of samples and IHRP were added at the above indicated final dilutions, and the inhibition reaction was started with the addition of Gly m 4-specific rabbit antiserum at a final dilution of 1:200,000. Inhibition reaction was carried out in a total volume of 100 µl/well and for 1h. For determination of maximal binding (B_0) of the Gly m 4-specific antibody, incubation buffer was added instead of antigen. For determination of non-specific binding (NSB) of the secondary antibody, wells were incubated with incubation buffer instead of soluble antibody and antigen. Thereafter, solid phase-bound Gly m 4 specific antibody was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (SigmaAldrich) at a dilution of 1:10,000. All incubations were carried out in total volumes of 300 µl/well and at ambient temperature. Between all incubation steps, microwells were washed four times with 100 µl/well washing buffer and emptied. Finally, after the addition of 100 µl/well substrate solution, solid phase-bound peroxidase catalyzed the formation of blue colored TMB product. The enzymatic reaction was stopped after 20 min incubation by the addition of 100 µl/well of 25 % sulphuric acid. Optical densities (OD) were read at 450 nm main (L1) and 630 nm reference (L2) wavelength, using a Spectramax 340 microplate reader (Molecular Devices, Munich, Germany). OD subtraction (L1-L2) and data processing were done using the microplate reader software (Softmax Pro v5.2, Molecular Devices) and as described elsewhere [16]. Briefly, OD values of samples or standards (B), and maximal antibody binding (B_0) were reduced by the OD of NSB, and % OD were calculated in relation to B_0 ($\% \text{ OD} = 100 \cdot (B - \text{NSB}) / (B_0 - \text{NSB})$). The sigmoidal standard curve, which resulted from semi-logarithmic plotting of % OD versus Gly m 4 standard concentrations, was fitted using the four-parameter-logistic (4PL)

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algorithm of the microplate reader software. Each of the rGly m 4 standards, NSB, and B₀ were run in duplicate wells. Each of the four serial dilutions of a sample extract was run in single wells. As described above, diluted extracts of the IHRP were run in duplicate and single wells, respectively. Investigated samples of active soy challenge meal, placebo, SPI and IHRP were considered positive if detected above the lower limit of detection (LOD, 2 ng/ml). Quantitative analysis was done for positive samples at or above the lower limit of quantification (LOQ, 5 ng/ml). LOD and LOQ were based on statistical calculation from duplicate analysis of NSB as described below. Moreover, at the LOQ, a maximum coefficient of variation (% CV) of 20, derived from the precision profile of the standard curve, was acceptable. ELISA runs were accepted if quantitative results of IHRP samples ranged within a maximum of 30 % variation between individual runs (data not shown). Investigated samples of active soy challenge meal, placebo, and SPI were quantified on the basis of each three individual extracts. Each extract was quantified on the basis of four serial dilutions (1:20, 1:50, 1:100, 1:250) in each single well analysis.

2.6 Statistical analysis of ELISA standard curves and triplicate sample extracts

To calculate an LOD of the Gly m 4 specific ELISA, the mean OD values and standard deviation (SD) of OD of NSB was calculated. The LOD, expressed as ng/ml of Gly m 4, was obtained by interpolating mean OD value plus three times SD of mean OD versus the standard curve. The LOQ, expressed as ng/ml of Gly m 4, was calculated by interpolating mean OD value plus six times SD of mean OD versus the standard curve. In addition, minimal requirement of precision at the LOQ was a % CV of 20 or less. % CV was calculated from duplicate analysis of individual standards. 4-parameter logistic (PL) curve fitting and statistical analysis of individual ELISA standard curves was done using Softmax Pro v5.2. Calculation of 95 % confidence bands of all valid ELISA standard curves within one ELISA lot, and box plot analysis of % CV of triplicate sample extracts was done using SigmaPlot for Windows version 13.0.

2.7 Gly m 4 specific immunoblot analysis

Immunoblot analysis of Gly m 4 in SPI (pilot batch M350033395) was done for the investigation of extraction buffer efficiency, using SDS-PAGE according to the method of Laemmli [17], semi-dry blotting according to Khyse-Anderson [18], and immunodetection based on Gly m 4-specific rabbit antiserum similar to Mittag et al. [4]. With each extraction buffer, triplicate protein extracts were prepared as described above for sample preparation for ELISA analysis, except for extracting 150 mg of sample material in 1.25 ml of extraction buffer. TT (as described above), PBST (PBS, 1 % Tween 20, pH 7.1), and IEF (isoelectric focusing buffer, consisting of 7 M urea, 2 M thiourea, 20 mM Tris, 65 mM dithiothreitol, pH 9), adapted from Mooney et al. [19], served as extraction buffers. SPI sample extracts, diluted 1:160 in electrophoresis sample buffer (10 mM Tris/HCl, 1 % sodiumdodecylsulphate, 0.5 % dithiothreitol, 0.01 % bromophenol blue, 1 mM EDTA, pH 6.8), were heated for 10 min at 99 °C, and loaded at 10 µl/lane into 1.0 mm thick PA-gels, consisting of a 5 % stacking gel and 15 % separation gel (BioRad MiniProtean cell III, Munich, Germany). Electrophoretic protein separation at 150 V was complete after bromophenol blue reached the bottom of the gel. Proteins were blotted at 0.8 mA/qcm for 60 min onto 0.2 µm pore size nitrocellulose membrane (Whatman Protran®, Schleicher & Schuell, Dassel, Germany). After 2 h of membrane blocking with TBS (tris-buffered saline, containing 50 mM Tris, 150 mM NaCl, pH 7.4) containing 0.3 % Tween 20, Gly m 4 was detected by subsequent 1 h incubations with Gly m 4-specific rabbit antiserum diluted 1:20,000 in incubation buffer (TBS, 0.05 % Tween 20, 0.1 % BSA, pH 7.4) and horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG (SigmaAldrich) diluted 1:100,000 in incubation buffer, respectively. After each incubation step, blot membrane was washed 4 times 5 min with TBS containing 0.05 % Tween 20. After addition of LumiGlo Reserve substrate (KPL, via Medac, Wedel, Germany), signals were recorded within 10 sec exposure using a chemiluminescence reader (FusionFx 7, Vilber Lourmat, Eberhardzell, Germany). Signal intensities were recorded with a FusionCapt Advance software (Vilber Lourmat) and signals were expressed in percentage to the most intense signals recorded.

2.8 Quantitative statistical analysis of FC meals used in the BASALIT trial

Statistical analysis for the Gly m 4 content of [a] the SPI batches and [b] the meal batches produced for FC applications within the BASALIT trial pre/post SCIT comprised two different types of analyses of variance (ANOVA). In all ANOVA analyses performed, the between-groups factor <SPI batches> was used, and referred to the six different UELZENA packages of soy powder/ flour used during the total course of the trial.

In analyses of dose levels 4 and 9 of the active meals (per meal batch, in total n=24) an additional within-group factor for repeated measures (at week 0, 6 and 12) was used to investigate potential changes of the allergenic potential of meals over time. I.e. courses of Gly m 4 measures at week 0, 6 and 12 weeks after preparation and shipment to the analytical laboratory were analysed. The first order interaction was included as well to identify potentially varying courses of Gly m 4 in time for different SPI batches. Neither differences between the different packages of SPI nor those between varying measures in time were hypothesized. The significance level was set to $\alpha=0.05$. To retain the hypotheses of comparable Gly m 4 contents, the observed p values had to be greater than α .

Calculations were done by SPSS V20, GLM procedure.

3 Results and Discussion

3.1 Extraction buffer selection and extraction efficiency

The selection of an appropriate ELISA extraction buffer and the investigation of the extraction efficiency were based on a pilot batch (M350033395) of soy protein isolate (SPI) that was also used for the ELISA in-house reference preparation (IHRP). IEF buffer was chosen as example of a denaturing buffer for total protein extraction (adapted from [19]), PBST was chosen as a buffer containing detergent, and TT buffer was chosen from a previous successful ELISA test development [16]. Immunoblot analysis was chosen to directly compare buffers containing denaturing (IEF) or surface active (PBST) with twitterionic (TT) agents because SDS-PAGE and semi-dry blotting was supposed to eliminate denaturing or surface active components. Using Gly m 4 specific immunoblot analyses, IEF buffer presented the highest yield of extractable and detectable Gly m 4 in triplicate extract analysis. Based on the assumption of complete Gly m 4 extractability in IEF buffer and

subsequent to normalization against the Gly m 4 amounts in IEF buffer, triplicate extraction yielded $100 \pm 13.8 \%$, $67.7 \pm 3.5 \%$, and $46.2 \pm 10.8 \%$ extraction efficiency using IEF, TT, and PBST buffer, respectively.

However, in ELISA analysis IEF buffer led to strong false-positive signals due to interference of the antigen-antibody binding of the maximal antibody binding B_0 in the absence of soluble Gly m 4 antigen (data not shown). Even at dilution 1:400 to 1:1600 of IEF buffer in ELISA incubation buffer, IEF buffer resulted in a false-positive signal equivalent to 0.00375 % Gly m 4, which corresponds to approximately 18 % of the amount of Gly m 4 present in the selected SPI batch (0.02128 %) (see below). Thus, IEF buffer was not applicable to routine batch analysis of SPI and soy challenge meals using ELISA. However, correct ELISA quantification of Gly m 4 in IEF buffer would allow determining extraction efficiency of the TT buffer in comparison to the IEF buffer. Hence, for elimination of unspecific false-positive signals of IEF buffer, the amount of Gly m 4 in IEF buffer extracts of SPI required quantification based on the method of standard addition with 0, 15 μ g, 30 μ g, and 60 μ g rGly m 4 spiked to the IEF-extracts which were diluted 1:400 and 1:1600, respectively. Regression resulted in excellent linearity with coefficients of determination (R^2) of 0.9994 and 0.9998 at IEF dilution 1:400 and 1:1600, respectively. In SPI pilot batch M350033395, Gly m 4 extracted using IEF buffer was determined at $0.02128 \pm 0.00397 \%$ (18.7 % CV) using ELISA quantification based on the standard addition method. The same SPI batch was quantified at 0.01574 ± 0.00188 (11.9 % CV) Gly m 4 after extraction in TT buffer, using ELISA without the standard addition method but according to the protocol described for routine batch analysis. Accordingly, TT buffer extracted Gly m 4 with an efficiency of $74.0 \pm 8.8 \%$ compared to IEF buffer. In summary, two independent analytical methods, Immunoblot and ELISA analysis, determined comparable extraction efficiencies of 67.7 % and 74.0 % of TT-buffer in comparison to IEF-buffer. On average, TT extracted $70.9 \pm 3.2 \%$ (4.5 % CV) of Gly m 4 compared to IEF buffer, as was determined by two independent methods. Hence, TT was selected as appropriate extraction buffer for routine analysis of active meal and SPI batches without the necessity of routinely applying the method of standard addition to all samples. Assuming complete solubility of Gly m 4 using IEF buffer, the amount of

Gly m 4 in SPI can be calculated from the ELISA results on the basis of TT-extracts, extrapolated by a factor of conversion of 1.41 ($\pm$ 0.06), which compensates for a 70.9 % extraction efficiency.

3.2 Reproducible ELISA characteristics

Within the 56 months period of trial duration, a total of 106 individual ELISA tests were run for the quantitative analysis of 24 individual batches of active soy challenge meals and placebo meals, and the SPI material used for preparation of active soy challenge meals. Depending on the available rGly m 4 for preparation of standard curves, two individual lots of ELISA tests were prepared on the basis of prealiquots of rGly m 4, rGly m 4 specific rabbit antiserum, and IHRPs. From 'lot 1' and 'lot 2', 61 and 45 individual ELISA tests were run, respectively. This corresponded to 13 and 11 quantified challenge meal batches, respectively. Figure 1 displays the mean ELISA standard curves and precision profiles of both lots of Gly m 4 inhibition ELISA. The narrow 95 % confidence bands of the rGly m 4 standard curves of both lots indicate high reproducibility of standards within each lot. ELISA 'lot 1' and 'lot 2' performed highly comparable having mean points of inversion of 13.2 ng/ml (20.7 % CV) and 21.1 ng/ml (16.0 % CV) Gly m 4, and mean Hill slopes of -1.2 (9.8 %CV) and -1.0 (5.4 % CV), respectively. Based on statistical analysis of replicate blank samples (NSB), the average LOD of 'lot 1' and 'lot 2' tests was 2.2 and 2.0 ng/ml Gly m 4, respectively. Moreover, the LOQ as derived from statistical analysis of NSB was determined at 5.1 and 5.0 ng/ml Gly m 4. At the LOQ, both ELISA lots retained the required precision of % CV $\leq$ 20 (figure 1 C, D) within the 95 % confidence band.

Intra-ELISA precision was determined for each one randomly selected batch of active meal dose levels 4 and 9, respectively. Each sixteen replicates of each one extract of dose levels 4 (1:20 dilution) and 9 (1:250 dilution) within each one ELISA run resulted in 12.74 and 13.58 % CV, respectively. Further, repetitive analysis of each one active meal 4 and 9, and of SPI within eight consecutive days resulted in an inter-ELISA variability of 6.6, 6.1, and 10.2 % CV, respectively. In summary, the Gly m 4 specific inhibition ELISA showed a robust and reproducible performance in 106 individual runs, and intra- and inter-ELISA variation was well below 20 % CV.

3.3 Quantitative ELISA analysis of active meal batches, SPI, and placebo

For a comparative ELISA analysis of active soy meal batches, suitable dose levels needed to be identified. SPI contained 88 % soy protein according to the manufacturer and an average of 0.01261 % extractable and detectable Gly m 4 using the applied tris-tricine-based sample extraction and ELISA protocols (table 2). Based on an average LOD and LOQ of 2 and 5 ng/ml Gly m 4 in ELISA, and considering the factors of dilution caused by extraction and serial dilution for measurements, Gly m 4 in active meal dose level 3 was detectable around the LOD, and Gly m 4 in active meal dose level 4 contained the lowest amount of Gly m 4 that would be quantified at or above the LOQ. Moreover, the active meal doses nos. 6 to 9 approximately contained the same percentage of Gly m 4 (table 1). For a comparative batch-to-batch analysis, active meal dose level 9 was chosen as a representative dose for high level Gly m 4 meals, and active meal dose level 4 was chosen as a low level quantifiable dose. The active meal dose levels 4 and 9 were analyzed for Gly m 4 at the time points 0, 6, and 12 weeks. In addition, placebo meal batches and SPI batches were analyzed at time point 0 weeks. Table 2 summarizes the quantified amount of Gly m 4 in all investigated batches of active soy meal dose levels 4 and 9, and of SPI at the various time points. Investigated placebo batches were negative, i.e. below the LOD. Each result of quantified Gly m 4 in active meal batches and SPI material was based on three independently prepared and analyzed extracts. As a measure for precision, the coefficient of variation (CV) of triplicate extract analysis is depicted in figure 2. In active soy meal dose level 9 batches and SPI batches, % CV of triplicate extract analysis was at or below 20 % within 95 % of all triplicates quantified. Active soy meal dose level 4 batches, which contained Gly m 4 nearby the limit of quantification, showed a CV at or below 20 % for approximately 80 % of all triplicate measurements. Only 5 % of triplicate measurements showed a CV of greater than 25 %. Thus, 95% of triplicate extract quantification of active meal dose level 4 showed a CV at or below 25 %. In summary, triplicate extract analysis allowed sufficiently precise quantification of the active meal dose levels 4 and 9, and of SPI. Based on ANOVA a), consistency of SPI batches throughout the whole BASALIT trial can be assumed, and the average Gly m 4-content quantified using ELISA can be calculated from the individual results obtained for SPI batches (details

below). Thus, in SPI, Gly m 4 was quantified at an average of 0.01261 % (table 2). Considering an additional correction factor of 1.41 for the determined extraction efficiency of 70.9 % of the TT-buffer used for all extracts, SPI contained 0.01778 % Gly m 4 (177.8 µg/g) on average. Based on this average Gly m 4-content of SPI and the amount of SPI according to the recipe, the calculated amount of Gly m 4 in dry powder of challenge meal (µg/g) is summarized in table 1. The comparison of Gly m 4 content in meal dose levels 4 and 9 based on ELISA quantification of prepared meal batches (table 2) versus Gly m 4 calculated from SPI on the basis of recipe and Gly m 4 quantified in SPI (table 1) reveals an average Gly m 4 recovery of 52 ± 10 % in active meal dose levels 4 and 9, using TT-extracts and ELISA analysis. Thus, the challenge meal matrix does have an additional impact on the recovery of Gly m 4. Independently, because of merely identical standardized sample matrix throughout all food challenges, the systematic quantitative error according to matrix effect can be neglected when investigating relative batch-to-batch consistency on the basis of comparative quantification. Figure 3 shows all data points (table 2) of relative quantification of SPI and 24 batches of active soy challenge meal dose levels 4 and 9 within a period of 56 months in comparison to the 95 % confidence and prediction bands, respectively. Figure 3 illustrates that quantification of the two selected dose levels 4 and 9, and of SPI is well separated and merely consistent over the entire period of 56 months. A slight increase of quantified Gly m 4 over time is observed for all samples. The slopes of linear regression were almost identical, indicating a comparable increase over time. Because no apparent differences between batches and time intervals were observed (details below), the slight increase of Gly m 4 quantification over time likely results from a minor systematic effect of the ELISA method.

3.4 Results regarding comparability for active meal batches and SPI, and between time intervals

As indicated in table 2, the active challenge meal batches were prepared from six different SPI batches. Between one and nine challenge meal batches were prepared from each of the SPI batches. ANOVA [a] was performed using the SPI batches as between-subject factor to identify possibly significant Gly m 4 differences during the course of the trial. The Gly m 4 contents of the different

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packages of SPI used did not differ significantly ($p=0.514$) but showed highly comparable Gly m 4 contents. ANOVA [b1] and [b2] analysed both - differences of SPI batches and potential timely effects regarding the Gly m 4 contents of the vials of meal dose level 4 resp. 9 (measured at weeks 0, 6, and 12) in parallel. Neither statistically significant differences of Gly m 4-content were identified between the batches of SPI ($p=0.128$) nor regarding the overall timely course and an interaction with the SPI batches ($p=0.864$ resp. $p=0.763$) were identified in [b1] results for dose levels 4. The latter would have referred to potentially varying courses in time dependent on the different SPI batches. ANOVA [b2] done in the same way with measures of dose level 9 vials surprisingly resulted in significant differences between batches of SPI ($p=0.027$) and nonsignificant but conspicuous overall effects in time ($p=0.076$), their interaction without remarkable findings ($p=0.369$). Given that no significant differences between batches of SPI were found in the analyses of SPI and active meal dose 4 vials, we consider these effects in meal dose 9 analysis vials not too meaningful. Possibly these results are rather a chance finding caused by the productional process of meals (which required very high precision at every step) and/ or certain inaccuracy of measurement than a systematic difference which is hardly to explain against the background of all results. In addition, no apparent differences in Gly m 4-contents over all batches can be seen between SPI, dose level 4 and dose level 9 vials in figure 3. In none of the investigated batches of dose levels 4 and 9, CV of quantified Gly m 4 at varying times (0, 6, and 12 weeks intervals) was greater than 25 %. In summary, Gly m 4-content in the batches of meal dose level 4 and 9 were stable in 24 different batches and within a period of 12 weeks for each batch and with, in average, less than 20 % mean coefficient of variation. A marginally higher % CV of active meal dose level 4, quantified around the LOQ of ELISA, versus dose level 9 batches (table 2) correlates well with the precision data of triplicate extract analysis as depicted in figure 2 and can be explained with a lower precision at the LOQ. Aggregated over all batches of meals, the following means [lower; upper 95 % confidence limits] of Gly m 4 were quantified using ELISA on the basis of TT-extracts:

- Vials of active meal dose 4: 0.000410 [0.000386; 0.000434] %,

- vials of active meal dose 9: 0.002164 [0.002051; 0.002278] %, and
- vials of SPI: 0.012610 [0.011741; 0.013478] %.

4 Concluding remarks

For verification of batch-to-batch consistency of provocation meals, a Gly m 4 specific inhibition ELISA was developed, validated, quality controlled, and applied in all batches of provocation meals within the BASALIT trial. Thus, 24 batches of active and placebo meals, including the underlying 6 batches of soy protein isolate, needed to be analysed for Gly m 4. Each batch of active meal required repeated analysis at week 0, 6, and 12 for verification of consistency during the individual 12 weeks shelf-life per batch. According to recipe, the differences in Gly m 4 concentration between the subsequent dose levels ranged between 2.42-fold (e.g. between levels 5 and 6) and 139-fold (e.g. between levels 2 and 3) in the dry powders of meals (table 1). To allow interpretation of differences in clinical reactivity of study subjects between the subsequent dose levels, the ELISA required adequate repeatability and precision. Hence to allow discriminating between dose levels having at least two-fold differences in Gly m 4 concentrations, the ELISA should meet precision with a coefficient of variation of $\leq 30\%$. In order to achieve adequate precision and comparability, we quality controlled the ELISA performance with in-house reference preparations. ELISA runs were accepted only if CV of IHRP quantitative results were at or below 30 % between runs. According to the validation data, intra- and inter assay precision were well below 20 % CV and triplicate extract quantification of samples was below 25 % CV for the great majority of samples tested. Taken together, the ELISA developed allowed quantification of challenge meal batches with a CV below 30 %. In summary, Gly m 4 was quantified consistently with a mean CV < 20 % both between the 24 soy challenge meal batches and within the individual 12 weeks of shelf-life per batch. Statistical analysis underlined these findings. Accordingly, the ELISA presented would also allow verification of the content of the individual allergen Gly m 4 in allergen products according to European Pharmacopoeia [20] which demands verification of the amount of individual allergen between 50 and 200 % in consecutive

batches of therapeutic allergen products. In the cocoa matrix of the challenge meal, Gly m 4 was recovered at approximately 50 %. For the comparative analysis of batch-to-batch consistency, this systematic quantitative error due to matrix could be neglected because of merely identical matrix composition. The inhibition ELISA developed presented an LOD and LOQ of 2 and 5 ng/ml, respectively. Meal dose level 4, having determined 7 ppm Gly m 4, was the lowest dose above LOQ that could be verified in the comparative analysis. The high dose level 9 having approximately 47 ppm Gly m 4 served as an additional comparator for the assessment of consistency. In the 6 SPI batches and in the meal dose levels 4 and 9 of all 24 soy challenge meal batches, consistency with mean % CV below 20 was determined experimentally and verified statistically. In general, it is thus assumed that all levels of challenge meal batches had consistent Gly m 4 contents between batches. The amount of Gly m 4 in SPI was determined at 178 µg/g (16.85 % CV). Thus, the level of Gly m 4 in SPI is comparable to Gly m 4 amounts found between 8 and 638 µg/g in various soybean lines [4, 7–9]. Provided that Gly m 4 monosensitisation predominates in birch-associated soybean allergy, to the best of our knowledge, this is the first human double blind placebo controlled food challenge study proving consistency of the relevant allergen content over the entire study period. Moreover, on the basis of the quantification of the relevant allergen Gly m 4, the individual allergic sensitivity of patients having birch-related and Gly m 4-mediated soy allergy may be related to the relevant allergen amount instead of total protein of the allergenic source, which will improve our basic knowledge in the pathomechanism of food allergy. In the baseline soy DBPCFC before immunotherapy, the median [interquartile range] cumulative eliciting dose for first subjective signs and objective symptoms were 0.7 g [0.2 – 4.7] and 4.7 g [0.7 – 24.7] total soy protein, respectively (Treudler R. et al., under revision. Provided that subjects with birch-related soybean allergy are monosensitised to Gly m 4, and considering 0.01778 % Gly m 4 and 88 % total soy protein in SPI, the median cumulative eliciting dose for first subjective signs and objective symptoms were 141 µg [40 – 950] and 950 µg [141 – 4991] Gly m 4, respectively.

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participation in the authorization procedure of the BASALIT trial and not authorized to advice or instruct any PEI employee involved in the authorization procedure.

Author contributions

Conceived study: AF, AS, JCS, RT, SV, TH. Designed and/or performed experiments: AF, SR, TH.

Analysed data: AF, SR, TH. Contributed reagents/materials/analysis tools: AF, JL, WMB. Wrote the paper: AF, JL, TH. (all author contributions in alphabetical order)

Abbreviations

ANOVA, analyses of variance; **DBPCFC**, double blind placebo controlled food challenge; **IHRP**, in-house reference preparation; **IMAC**, immobilized metal ion chromatography; **LOAEL**, lowest observed adverse effect level; **4-PL**, 4-parameter logistic; **NSB**, non-specific binding; **SPI**, soy protein isolate; **TBS**, tris-buffered saline; **TMB**, 3,3',5,5'-tetramethylbenzidine; **TT**, tris-tricine;

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Tables

Table 1. Composition of the dry powder of active soy challenge meals, including calculated amounts of SPI soy protein according to manufacturer, and calculated Gly m 4 content according to ELISA analysis of Gly 4 in SPI ingredient. (* calculated on the basis of 0.01778 % Gly m 4 in SPI, which was derived from 0.01261 % mean Gly m 4/SPI quantified in ELISA and corrected for 70.9 % TT-extraction buffer efficiency).

Dose level no.	1	2	3	4	5	6	7	8	9
g total soy protein/dose	0.000044	0.00044	0.05	0.15	0.5	1.5	2.5	5.0	15,0
g dry powder of each dose	4.200	5.200	4.257	4.371	4.768	5.905	10.741	21.482	64.445
% SPI (88% total soy protein)/ dry powder dose	0.001165	0.00961	1.335	3.901	11.92	28.86	26.45	26.45	26.45
% total soy protein /dry powder dose	0.001023	0.008461	1.175	3.432	10.49	25.40	23.28	23.28	23.28
µg Gly m 4 /gram dry powder dose (ppm),	0.0021	0.017	2.37	6.94	21.2	51.3	47.0	47.0	47.0

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calculated*									
µg Gly m 4/ dose, calculated*	0.009	0.09	10.1	30.3	101	303	505	1010	3031

Table 2. Quantified % Gly m 4 in SPI batches at week 0 and in active meal batches dose 4 and 9 at time points 0, 6, and 12 weeks, respectively.

batch no.	ID of meals batch	meal level - week						ID of SPI batch	SPI - 0
		4 - 0	4 - 6	4 - 12	9 - 0	9 - 6	9 - 12		
1	20100129	3,63041E-04	2,33418E-04	3,16385E-04	1,36536E-03	1,74107E-03	1,71291E-03	M350034053	n.d.
2	20100529	3,49669E-04	3,90033E-04	3,51086E-04	1,61784E-03	1,72785E-03	1,58232E-03	M350034053	9,257611E-03
3	20100726	2,83545E-04	2,45134E-04	2,38353E-04	1,72506E-03	1,56711E-03	1,38762E-03	M350034053	1,112897E-02
4	20100927	3,12442E-04	3,46095E-04	3,66733E-04	1,70819E-03	2,00821E-03	1,82293E-03	M350034053	1,320064E-02
5	20101006	2,70691E-04	3,85740E-04	3,48012E-04	1,60948E-03	2,45834E-03	1,85479E-03	M350034053	1,280402E-02
6	20110126	5,31092E-04	4,81150E-04	5,23913E-04	2,29600E-03	2,70139E-03	2,70642E-03	M350034053	1,460714E-02
7	20110530	4,28501E-04	3,77971E-04	3,44916E-04	2,02972E-03	2,10193E-03	2,35371E-03	M350040448	1,239457E-02
8	20110725	3,89728E-04	3,72352E-04	5,72135E-04	1,75682E-03	2,53822E-03	2,82230E-03	M350040448	1,247359E-02
9	20111004	2,59808E-04	5,24968E-04	4,61475E-04	1,59610E-03	2,38255E-03	1,99905E-03	M350040448	9,588320E-03
10	20111018	3,90372E-04	n.d.	3,87596E-04	1,80914E-03	n.d.	1,97146E-03	M350040448	1,103197E-02
11	20120102	4,42009E-04	5,33903E-04	3,52027E-04	2,13592E-03	2,44807E-03	2,08164E-03	M350040448	1,298677E-02
12	20120319	4,62073E-04	3,69121E-04	3,48882E-04	2,15207E-03	2,07721E-03	1,77489E-03	M350040448	1,719377E-02
13	20120507	3,76103E-04	5,36927E-04	6,30875E-04	1,88849E-03	2,43327E-03	2,43111E-03	M350040448	1,405330E-02
14	20120719	3,87867E-04	3,42273E-04	5,26525E-04	1,94982E-03	2,01486E-03	2,45727E-03	P440032450	1,422990E-02
15	20120801	4,94403E-04	4,04931E-04	4,21414E-04	2,19051E-03	2,15577E-03	1,91173E-03	P440032450	1,206759E-02
16	20120814	5,00971E-04	3,99930E-04	4,32668E-04	2,30306E-03	1,93150E-03	2,24282E-03	P440032450	1,419120E-02
17	20120830	4,35880E-04	4,51536E-04	4,08893E-04	2,17090E-03	2,38255E-03	2,02583E-03	P440032450	1,129171E-02
18	20121002	4,92744E-04	3,43624E-04	4,18865E-04	2,24522E-03	1,98330E-03	2,19295E-03	P440032450	1,051020E-02
19	BAS4212Q0910/BAS4212M0910	4,03590E-04	4,08562E-04	3,21399E-04	2,00747E-03	2,03822E-03	1,91188E-03	M350044853	1,018742E-02

20	BAS0213A0910/BAS0213B0910	4,37736E-04	4,01857E-04	4,86230E-04	2,09910E-03	1,95555E-03	2,51382E-03	M350044853	1,263730E-02
21	BAS3113D0919/BAS3113E0910	3,29018E-04	4,65206E-04	4,11168E-04	2,14981E-03	2,43751E-03	2,32884E-03	M350044853	1,036093E-02
22	BAS4113E0910/BAS4113F0910	3,20318E-04	5,12269E-04	4,71363E-04	2,46843E-03	2,49063E-03	2,75094E-03	G010037922	1,208258E-02
23	BAS0214G0910/BAS0214H0910	4,72031E-04	4,52187E-04	4,30747E-04	2,39715E-03	2,45979E-03	2,93571E-03	ML100-26962-060114	1,474790E-02
24	BAS2414K0910/BAS2414L0910	4,98622E-04	4,75623E-04	4,48907E-04	2,42463E-03	2,55376E-03	2,17278E-03	G010037922	1,699112E-02
Mean		4,01344E-04	4,11079E-04	4,17524E-04	2,00401E-03	2,19951E-03	2,16441E-03		1,260950E-02
N		24	23	24	24	23	2,40000E+01		23
Std.Dev.		7,87747E-05	8,19559E-05	8,90191E-05	3,00195E-04	3,07883E-04	4,03407E-04		2,12416E-03
% CV		19,63	19,94	21,32	14,98	14,00	1,86382E+01		16,85

n.d. not determined

Figure legends

Figure 1. Mean standard curves of ELISA 'lot 1' (A) and ELISA 'lot 2' (B), and precision profiles of ELISA 'lot 1' (C) and ELISA 'lot 2' (D). Mean standard curves and precision profiles of both lots were derived from 61 and 45 individual ELISA runs, respectively.

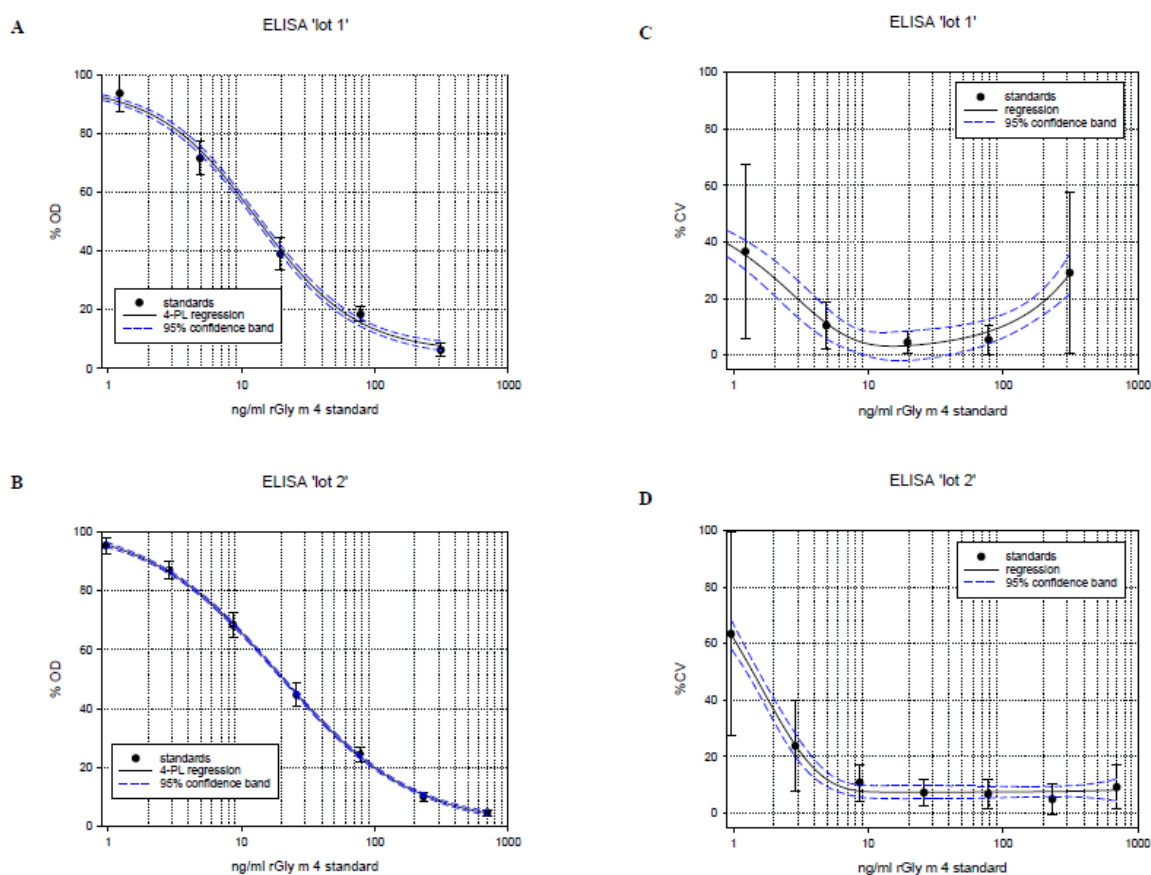


Figure 1.

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Figure 2. Precision of triplicate extract analysis per sample (1, active meal level 4; 2, active meal level 9; 3, SPI). Upper and lower lines of boxes indicate 75th and 25th percentiles, upper and lower whiskers indicate 90th and 10th percentiles.

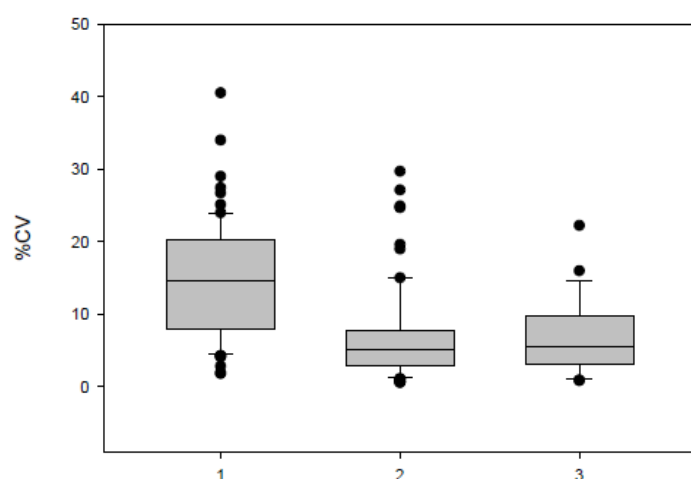


Figure 2.

Figure 3. ELISA Quantification of Gly m 4 in 24 challenge meal batches and SPI over a time course of 56 months. Each SPI batch was quantified at week 0 (filled diamonds); level 4 (filled circles) and level 9 (filled triangles) challenge meal batches were each quantified at weeks 0, 6, and 12. Dotted and straight lines indicate 95 % confidence and 95 % prediction bands, respectively.

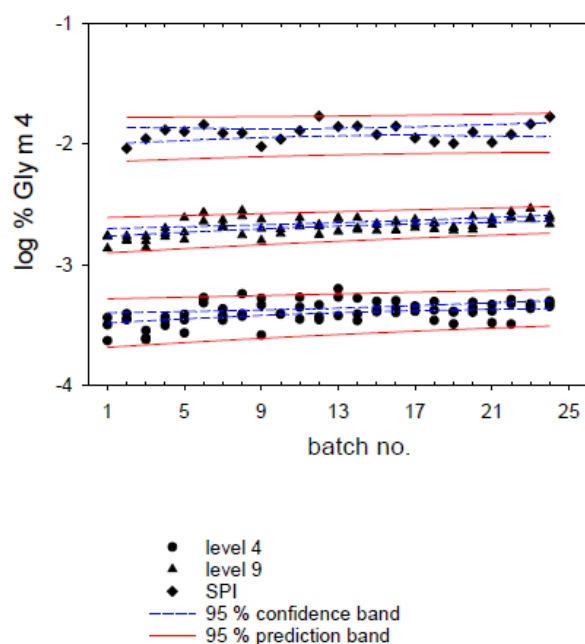


Figure 3.