**Supplementary to:**

**Development of a hypo-allergenic recombinant parvalbumin for first-in-man SCIT of fish allergy**

**Materials and methods**

*Assessment of allergenic activity: basophil histamine release test*

Histamine release (HR) from basophilic granulocytes was performed as previously described (1). In brief, peripheral blood mononuclear cells (PBMCs) from selected buffy coat blood (healthy non-atopic donors, anti-IgE responding cells) were isolated by Lymphoprep-gradient centrifugation. After intensive washing with saline, cell-bound IgE was removed from cells by a wash-centrifugation step using a stripping buffer containing 20 g/L potassium chloride and 0.37 g/L sodium hydrogen phosphate at pH 3.55. The pH was returned to 7.4 by wash in Pipes (piperazine-N,N′-bis(2-ethanesulfonic acid) buffer. PBMCs were then incubated with sera from fish allergic patients (n=24) or a healthy control for 1 hour at 37°C to passively sensitize basophils. The erythrocyte fraction from the density-gradient centrifugation was washed in Pipes buffer and reconstituted with Pipes buffer to a volume 1.5x the original blood volume. After sensitization, the PBMCs were given 3 ml erythrocyte-suspension. Six dilutions (~100 μg to ~1 ng/ml) of nCyp c 1, rCyp c 1 or mCyp c 1 were prepared in allergen-coated glass fiber microtiter plates (Reference Laboratory, Copenhagen, Denmark) and incubated with PBMC-erythrocyte suspension under releasing conditions at 37°C for 60 minutes. Anti-IgE was used as positive control. Released histamine bound to the glass fibres was coupled to 0.05% (v/v) o-phtahaldialdehyde, stabilized by 0.6% (v/v) HClO4 and measured fluorometrically as described in Stahl Skov et al (2). Results were expressed in ng/ml of released histamine.

*Immunogenicity assessment: Laboratory animals and human PBMCs*

Immunization and plasma collection as described in the main paper. This was part of a larger experiment and the pre-immune serum was pooled from all rabbits (n=20). Plasma was recalcified and IgG antibody titers against rCyp c 1 were measured by ImmunoCAP using rCyp c 1 ImmunoCAPs (f355). Dog allergen ImmunoCAPs (e5) were used to assess unspecific binding. A β-galactosidase-conjugated antibody to rabbit IgG was used to detect bound IgG (not commercially available; a kind gift from Thermo Fisher Scientific). Since no standard curve was available for measuring rabbit IgG, the standard curve for human IgE was used for expression of results in arbitrary units.

PBMC purification as described in the main paper. PBMCs were cultured at 1\*106 cells per ml in 0.5-2 ml RPMI 1640 medium (Sigma, Saint Louis, US) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 1 mM L-glutamine (Invitrogen, Carlsbad, US), 50 M β-mercaptoethanol and 5% (v/v) human AB serum. PBMCs were cultured with medium control, 10 µg/ml phytohaemagglutinin (PHA) (Sigma, Saint Louis, USA); 2, 10 or 50 µg/ml tetanus toxoid (SSI, Copenhagen, Denmark); 2, 10 or 50 µg/mL rCyp c 1 or mCyp c 1 for seven days. At day seven, the cultures were harvested and stained with anti-CD4-PC5 or -PC7 (OKT4, Biolegend, San Diego, USA) at room temperature for 15 min in the dark and analyzed by flow cytometry. Proliferation of CD4+ T cells in response to antigen stimulation was determined using FlowJo (Treestar, Ashland, USA). From each patient the PHA culture was used to set three proliferation regions around the 0-2, 3-5 and 6-9 divisions per cell. Antigen-specific proliferating T cells were identified to be in the “6-9” region. PBMCs from each patient should be stimulated with all relevant allergens in standard culture medium. A minimum of 20,000 events should be collected from each culture condition. There should be detectable proliferation (>20%) of CD4+ T cells in the culture stimulated with PHA. This meant of the 30 cod allergic patients analyzed, after quality control, 14 samples could be used for data analysis. In the figures we depicted the % allergen-specific cells proliferating as % of the negative control cells for each patient in the “6-9” region. This outcome was used because percentages of cells dividing under the negative control conditions were individually quite different.

*1D/2D PAGE*

All reagents were of analytical grade and purchased from Sigma Chemical Co, Dorset, UK unless otherwise stated.

SDS-PAGE analysis was performed under reducing and non-reducing (oxidised) conditions on a NuPAGE 4-12% Bis-Tris gel according to the protocol of the manufacturer (Invitrogen, Paisley, UK), dithiothreitol (DTT; 50mM) was incorporated as reducing reagent for samples run under reducing conditions. The gels were fixed in methanol (50% [v/v]), water (40% [v/v]) and glacial acetic acid (10% [v/v]) for 2hrs and stained with SimplyBlue SafeStain (Invitrogen) according to the manufacturers’ instructions. The gels were scanned using a BioRad GS800 densitometric scanner using the QuantityOne software package, (Bio-Rad, Hertfordshire, UK).

First dimension isoelectric focusing (IEF) was performed on 7 cm IEF strips (pH 3-11 non-linear, GE Healthcare, Buckinghamshire, UK). Aliquots of protein (20μg) were dialysed against water (MilliQ grade) using micro dialysis apparatus (Slide-A-Lyzer MINI Dialysis Units, 3.5kDa exclusion limit, Thermo Fischer Scientific, Leicestershire, UK) for 1 hour, before being loaded onto the IEF strip using the overnight rehydration technique according to the manufacturer’s instructions. Following strip rehydration, IEF was performed on a Ettan IPGphor electrophoresis unit (GE Healthcare) with a program consisting of 30’ 300V, a gradient to 1000V over 30’, a gradient to 5000V over 80’ followed 25’ at 5000V (total run time exposure of 6.5KVh).

The focused IEF strips were immediately reduced at room temperature (RT) for 10 minutes in 5mL of buffer (56% [v/v] Tris Acetate Equilibration Buffer (Genomic solutions, Cambridgeshire, UK,) 1.25% [w/v] sodium dodecyl sulphate, 3M Urea, 4% [w/v] CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), containing 10mg/mL DTT. Reduced strips were alkylated at RT for 10 minutes in 5mL of the same buffer containing 25 mg/mL iodoacetamide (GE Healthcare). Reduced and alkylated strips were subjected to second dimension SDS PAGE on a NuPAGE 4-12% Bis-Tris precast zoom gel. The gels were fixed as before then stained in 40ml SYPRO for 4 h (120rpm) at RT. After de-staining (10% [v/v] methanol:6% [v/v] acetic acid:84% [v/v] water) the gels were imaged using a Pharos FX Plus molecular imager, fluorescence scanner using the QuantityOne software package, (Bio-Rad).

*Proteomic analysis*

Gel plugs were removed from protein spots visualized either using SYPRO staining using a robotic system (Genomic Solutions Investigator ProPic apparatus), or using Coomassie blue staining with manual removal of the region of interest using a clean 5mL plastic pipette tip. Gel plugs were automatically digested with sequencing grade trypsin using a Genomic solutions ProGest Apparatus (Genomic Solutions, Cambridgeshire, UK). Gel pieces were conditioned with two 20 min incubations in 200 mM ammonium bicarbonate (ABC) in 50% (v/v) acetonitrile (50 µl) followed by 10 min incubations with acetonitrile alone (50 µl). The gel pieces were then conditioned for 15 min with 25 mM ABC (50 µl) followed by 10 min in acetonitrile (50 µl). Cysteine residues were then reduced by treating the gel pieces with 10mM DTT in 50mM ABC (50 µl) for 30mins at 60°C then alkylated by treating the gel pieces with 100mM iodoacetamide (50 µl) for 30mins. The initial pH equilibration steps with 200 mM ammonium bicarbonate (ABC) in 50% acetonitrile (50 µl) followed by 10 min incubations with acetonitrile (50 µl) were repeated before the gel plugs were conditioned for 15 min with 25 mM ABC (50 µl) and then shrunk by 10 min in acetonitrile (50 µl). A final 5 min incubation of acetonitrile (50 µl) preceded the trypsin digestion at 37 °C for 3 hours using 50 ng (5 µl per well) sequencing-grade porcine trypsin (Promega, Southampton, UK) dissolved in 25 mM ABC. Digestion was stopped and peptides extracted using formic acid (5 %; 5 µl per tube). Tryptic peptides were then analysed by MALDI TOF mass spectroscopy using a Reflex III MALDI/ToF (Bruker UK Ltd, Coventry) with Scout 384 ion source using a nitrogen laser (wavelength 337nm) to desorb/ionise the matrix/analyte material from the sample substrate. All spectra were acquired in a positive-ion reflector. The acceleration voltage was set to 25 kV, the reflection voltage was set to 28.7 kV, ion source acceleration voltage to 20.9 kV, and the reflector-detector voltage to 1.65 kV. Calibration was carried out using a set of peptide standards of approx. conc. 1 pmol/ul from spots adjacent to the samples.Resultingpeptide mass fingerprints were searched against Mascot (Matrix Science London, UK) using the complete database to detect plant, animal and bacterial proteins).

*Size-exclusion chromatography*

Size-exclusion chromatography was performed on each sample (75 μL) on a column of Superdex 75, 10/300 attached to a ÄKTA FPLC (GE Healthcare) equipped with a variable wavelength detector for the initial and three month storage samples using Unicorn 5.01 software. The column was equilibrated in sodium phosphate buffer pH 7.4, containing 150 mM NaCl and 0.01% (w/v) sodium azide at 0.5 mL/minute. The eluent was monitored at 220 nm. Relative molecular mass (really equivalent hydrodynamic volumes) was determined from a calibration using a sigmoid curve calibration performed using GraphPad Prism version 5.01 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com)..

*Circular dichroism spectroscopy (CD)*

Samples (60 μL) were diluted with 60μL of HPLC grade water (NanoPure Diamond, Barnstead, Thermo Fischer Scientific). Spectra were acquired from 260 to 190 nm at 0.5 nm resolution at a scan speed of 100nm/minute in a 0.5 mm quartz cell, six spectra were accumulated to produce the mean spectra using a Jasco J-710 spectropolarimeter (Jasco, Essex, UK). Molecular weights and number of amino acids in each protein were calculated from the peptide sequence of each protein to calculate the molar elipticity. CD secondary structure analysis was performed using CDPro (Colorado State University, Colorado, USA).

*Reverse phase high performance liquid chromatography* (*RP-HPLC)*

Samples (25 μL) were analysed on a reverse phase HPLC column (Jupiter C4, 300Å, 5μm, 4.6 x 250 mm, Phenomenex, Cheshire, UK) attached to a Summit HPLC system equipped with a variable wavelength detector and using Chromeleon 6.80 software (Dionex, Surrey, UK). The column was held in a thermostatic column oven at 20oC using a solvent gradient using solvents A (99,9% (v/v) water containing 0.1% (w/v) trifluoroacetic acid) and B (90% (v/v) acetonitrile,10%(v/v) HPLC water containing 0.085% trifluoroacetic acid w/v) at a flow of one mL/min. The gradient profile was; 5 min at 99% (v/v) A ; 55 min at 50% (v/v) A ; 15min at 0% (v/v)A, after which 15min 0% (v/v) A and re-equilibration at 99% (v/v) A (20 min) in preparation for the next sample. The eluent was monitored for protein at 220 nm.

*MALDI –TOF mass spectroscopy for intact mass analysis*

Sample (5 μL) was mixed (1:1 v/v) with a saturated sinapinic acid (Sigma-Aldrich, Dorset, UK) matrix in 30% (v/v) acetonitrile, 0.1% (v/v) trifluroacetic acid (TFA). The target plates used were polished stainless steel (Bruker Daltonics, Coventry, UK). Samples were prepared in the presence of 5 mM Tris(2-carboxyethyl)phosphine (TCEP). Approximately 0.5 μL of sample/matrix mixture was spotted onto the MALDI target and dried in air. The MALDI-MS measurements were performed using a Bruker UltraFlex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a pulsed N2 laser (λ = 337 nm, frequency 10 Hz). Whole protein spectra were recorded over the 2000-12000 Da range in linear mode at an accelerating voltage of 25 kV by averaging of at least 300 individual laser shots. The concentration of each protein solution was adjusted to give peak intensity similar to that of the calibrators used. Calibration was performed using ACTH (adrenocorticotropic hormone), somatostatin, ubiquitin, insulin and myoglobin.

*Accelerated stability studies of mCyp c 1*

All samples were stored for 3 and 6 months at different temperatures (temperature over experiment duration indicated between brackets): -80ºC (Ultra cold laboratory freezer, Sanyo VIP series -86 model MDF U71V [-79 to -80ºC]), -30ºC (Standard refrigerator, Meikama Lumimies freezer [-35 to -28ºC]), 4 ºC (IFR cold room [+7 to +3ºC]), 25ºC (Laboratory oven, Memmert oven model UM100 [24 to 27ºC]) and 40ºC (Laboratory Incubator, Gallenkamp economy incubator [38 to 41oC]). The temperatures of the stored samples were checked on a weekly basis using a thermocouple thermometer (Hanna instruments model Hi 93531 (Michigan, USA) equipped with a Fe/CuNi electrode, BS 4937J). Samples were placed in screw capped Eppendorf vials, stored upright and tightly wrapped with plastic food wrap (Clingfilm), sealed inside a zip lock plastic bags prior to placing at the selected storage temperatures. Prior to analysis all the samples were allowed to equilibrate to RT and gently mixed by inversion on a rotary wheel mixer for five minutes at 10 rpm (Stuart SB3 rotator, Bibby Scientific Limited, Staffordshire, UK). An aliquot of each sample (10 μL) was removed and stored frozen at -80ºC for subsequent MALDI mass spectroscopy. Samples were then stored at 4ºC for up to 5 days before analysis.

*Production of non-GMP rCyp c 1 and GMP mCyp c 1*

Cloning and expression of recombinant rCyp c 1 and mCyp c 1

For expression of both the recombinant parvalbumin rCyp c 1 (EMBL accession no. AJ292211) and mCyp c 1 (4) under the control of T7 promotor, the pET-28b(+) vector (Novagen, USA) was used. Vector construction (codon optimization, gene synthesis and subcloning was performed by ATG:biosynthetics GmbH (Merzhausen Germany). To manufacture the master cell bank (MCB) for both non-GMP rCyp c 1and GMP mCyp c 1 chemically competent *E. coli* BL21(DE3) cells (5) (Novagen, USA) were transformed with the expression plasmid and incubated overnight at 37°C. Single colonies were tested for rCyp c 1 and mCyp c 1 expression by SDS-PAGE. The best performing clones were selected for establishment of the GMP Master Cell Bank for mCyp c 1. For GMP-production of the MCB, an aliquot from the glycerol stock was cultured until OD600 = 1 – 2. Glycerol was added to a final concentration of 15%, the cultures were aliquotted and frozen at -75 ± 10°C. The mCyp c 1 was produced at the manufacturing site of Biomay in a GMP clean room, following GMP guide lines. In contrast, the rCyp c 1 was produced similarly, but not in a GMP certified clean room.

Fermentation and primary recovery of the recombinant rCyp c 1 and mCyp c 1

Both the rCyp c 1 and the mCyp c 1 were manufactured by utilizing a fed-batch fermentation technology. The culture medium was synthetically defined, i.e. consisting of macro and micro elements and glucose, but without animal derived compounds, without complex compounds and without antibiotics. One MCB vial was cultured in a shake-flask until OD ~ 1 was reached. rCyp c 1 and mCyp c 1 expression was initiated by addition of IPTG (to a final concentration of 0.8mM).Production culture was then carried out in a stainless steel fermentor (BIOSTAT C15-3 MO, Sartorius, GMP clean room class D) equipped with a process control system (MFCS/win, Sartorius). The fermentation process was operated in fed-batch. Fermentation broth was harvested and cell biomass was separated by centrifugation at 5500x g (SRC5B, Sorvall) with fixed angle rotor (–F10S). Harvested wet cell biomass was aliquoted into sterile polypropylene bags and stored at -75°C.Frozen Biomass was resuspended in alkaline tris buffer and thawed using a water bath. Suspended cells were disrupted by using a homogenizer (Silent Crusher, Heidolph Instruments). The crude cell homogenate was subjected to a batch centrifugation step using a high speed centrifuge at (5500x g). For purification and clarification of the supernatant, contaminating proteins and turbid matters were precipitated using Poly(ethylene imine)-solution and separated by centrifugation as before. The clarified supernatants were collected and pooled for further downstream purification.

Downstream (Purification) Process of rCyp c 1 and mCyp c 1

Chromatography steps were carried out on an ÄKTA Pilot chromatography system (GE Healthcare). For purification, a three-step purification process was applied, comprising anion exchange (Q Sepharose FF), hydrophobic interaction I (Phenyl Sepharose FF) and hydrophobic interaction II (Phenyl Sepharose HP). Aliquots of 45mL fractions were collected with ÄKTA fraction collector (GE-Healthcare) and stored at +4°C until pooling. Fractions containing purified product according in-process control with SDS-PAGE (Biorad systems) were selected for pooling. The pool of the second hydrophobic interaction chromatography step was subjected to ultra-/diafiltration. The pool was first concentrated and afterwards diafiltrated against formulation buffer. After a 0.2 µm filtration the actual protein concentration was measured to be 1.6 mg/mL (based on absorbance at A280nm) and the protein was aliquotted. The final target scale of GMP manufactured drug substance mCyp c 1 was >400 mg drug substance. One GMP drug substance manufacturing batch was performed. All the downstream processing work was done in clean room class C, open product steps in laminar work flow class A or class B, and filling steps in class A.

*Characterization of rCyp c 1 and mCyp c 1*

Product-related purity and impurities of rCyp c 1 and mCyp c 1

In order to obtain a pure product the product related impurities were analysed by Coomassie-stained SDS-PAGE under reducing and non-reducing conditions. Proteins were visualised by Coomassie Blue staining and evaluated by densitometric analysis. In addition Reversed Phase chromatography was performed by an Agilent 1200 HPLC station with acetonitrile complemented with 0.1% TFA as solvent and UV detection at 280 nm.

Circular dichroism and size exclusion analysis of purified rCyp c 1 and mCyp c 1

### Circular dichroism measurements were performed in PBS on a Jasco J-715 spectropolarimeter with protein concentrations between 12.3–24.0 μM using a 1 mm path-length quartz cuvette (Hellma) equilibrated at 20°C. Spectra were recorded from 197 to 260 nm with 0.2 nm resolution at a scan speed of 50 nm/min. The final spectra resulted from the average of three scans, were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions and evaluated according to Jedrychowski (6). Aggregation behavior of both proteins was analysed by Size Exclusion chromatography (HP-SEC) on an Agilent 1200 HPLC station by applying 5 mM sodium phosphate buffer, 0.05% sodium azide pH 7.4 as solvent and UV detection at 280 nm.

Western Blotting and Mass Spectrometry

For further characterisation of the mCyp c 1 drug substance Mass Spectrometry and Western Blot were applied. Electro-blotting was done on BioTrace NT Membrane (Pall). Protein-specific mouse polyclonal antibodies were applied as primary antibodies. Detection was done by alkaline phosphatase (AP) conjugated rabbit anti-mouse IgG antibodies (Sigma Aldrich). Identity of rCyp c 1 and mCyp c 1 was confirmed by comparison of the mass determined by ESI-QTOF mass spectrometry with the theoretical (calculated) mass of rCyp c 1 and mCyp c 1.

RP-HPLC analysis, LC/ES-MS analysis of rCyp c 1 and mCyp c 1

For further characterization of rCyp c 1 and mCyp c 1 and identification of the four, major peaks detected by RP-HPLC analysis, LC/ES-MS was outsourced to SGS M-Scan GmbH (Germany).

Both rCyp c 1 and mCyp c 1 were tested for microbiological contamination (in accordance to Ph. Eur. 2.6.12) by the membrane filtration method. The proteins were sequenced N-terminally with Edman degradation (outsourced to Eurosequence, Groningen, The Netherlands). Endotoxin content of the GMP mCyp c 1 was determined using the Endosafe-PTS System (Charles Rivers).

*Formulation of mCyp c 1 drug products and real-time stability testing*

Formulation and adsorption

The whole production process of the placebo and diluent are comparable to the production process of the drug product, which is described below.

The formulation was performed in a class C environment:

The mCyp c 1 was thawed over night by placing it at 2°C – 8°C in a refrigerator. The next morning, when the solution was thawed, an in process control (IPC) was taken for measuring the protein concentration. NaCl (Merck, VWR International, The Netherlands) and liquid phenol (Sigma Aldrich Chemie, The Netherlands) are added (final concentration 0.9% and 0.5%, respectively) and subsequently sterile filtration (using a mini kleenpak fluorodyne II filter of 0.2 µm, Pall) was performed in a class A environment. All materials which have contact with the product were washed and sterilised according to validated processes. The formulated and sterile filtrated mCyp c 1 solution was adsorbed onto aluminium hydroxide (alum), Alhydrogel, at 2 mg/ml and aseptically added to the solution. The concentration was based on the highest concentration of Cyp c 1 that could be absorbed to this amount of alum (which did not exceed the maximum allowed daily dose of alum). The suspension was filled into sterile 50 ml vials.

Filling

Filling was performed in a class A environment:

The mCyp c 1 was filled into 6 ml vials (6R, Aluglas B.V, The Netherlands). The 6R vials were rinsed with water for injection. The vials are depyrogenised in dry heat for 30 min at 250°C or equivalent. The rubber closure and the aluminium caps were sterilised with moist heat for 15 minutes at 121°C or equivalent. The 50 ml vials containing the suspension were homogenized by inverting the vials several times. The vials were filled manually using a syringe. Each vial was filled with 3 ml of mCyp c 1. During filling the 50 ml vials were inverted regularly to ensure homogenous dispensing.

After filling of the batch, samples were taken on which full analysis was performed; The appearance and color were visually inspected, pH was measured, the aluminium hydroxide content was determined by atomic absorption spectrometry (Proxy Laboratories, The Netherlands), total protein content was determined by the Kjeldahl method (Dutch Vaccin Institute, The Netherlands), the protein in supernatant was measured with the Bradford assay (reagents: Biorad, USA; BSA standard: Pierce, USA), sterility was checked with the direct inoculation method and the phenol content was determined.

Labeling and packaging

Labeling and packaging were carried out in a non-classified environment:

The vials were labeled and packed in a box. Labeling and packaging was performed batch wise. The final packed product was stored at 2°C – 8°C.

Stability testing

During stability testing of the product, at several timepoints (0, 3, 6, 9, 12, 15 and 20 months) the full analysis as described above under “filling” was performed. In addition an inhibition ELISA based on rabbit IgG against the drug substance has been developed as a surrogate for a true potency determination to allow stability studies of the drug product according to the Guideline on Allergen Products. The plate was coated overnight with mCyp c 1 (62 ng/well) in coating buffer (0.1 M NaHCO3, 0.033 M Na2CO3, pH 9.5). The next day plates were washed 3x in PBS/0.05%TWEEN and blocked with 100 µl/well TTF (TBS, 0.05% TWEEN20, 10% FCS), 1 hour at RT. A dilution range of the samples from the stability study (Al(OH)3- mCyp c 1) starting from 1.04 mg/ml to 5.8x10-6 µg/ml and a dilution range of the standard (mCyp c 1, kept at -80˚C, thawed fresh every time) starting from 1.55 mg/ml to 2x10-7 µg/ml was mixed in TTF buffer with a dilution of rabbit anti- mCyp c 1 antibodies and left at room temperature for 2 hours. After this, the samples were pipetted in triplo and incubated for an hour before washing as before. Then the plates were incubated with swine anti-rabbit-HRP (Dako, Denmark, diluted 1:2000 in TTF) for an hour. After washing 3 times, the TMB substrate (eBioscience, USA) was added, the plates were incubated in the dark for 10 minutes and absorbance was measured at A 450-655. Soft Max Pro software was used to make the curves and calculate an IC50 (the concentration at which the samples are 50% inhibited) with the so-called ‘4-parameter method’. The mean IC50 is the mean of measurements in triplicate, done on three consecutive days.

*Toxicity studies*

Acute

For acute toxicity studies rodents (specific pathogen free (SPF) mice (HsdWin:NMRI, Harlan, The Netherlands) and non-rodents (SPF) New Zealand white rabbits (Lidköpings kaninfarm, Sweden) were used. Non-rodent species were used to confirm the observations made in rodents. The isolation and/or acclimatization period in the test facility was 32 to 35 days and only healthy animals were accepted into the study. The “Directive 2010/63/EU of the European parliament and of the council (22 September 2010) on the protection of animals used for scientific purposes” were followed for the care and use of the animals.

In the preliminary sighting study the dose of 10 or 0.3 mg/kg body weight (bw) of test item mCyp c 1 was chosen as a safety margin over the maximum dose in the draft protocol for the first-in-man safety study (x-fold on a bw basis).

Repeated dose

Following the protocol in the main paper, mice were injected sc with the test substance once a week, over a period of six months (25 doses, animals were sacrificed one week after the last dose of the test item, on study week 26). The dose volume was maximally 5 ml/kg and was based on real-time individual body weight data. Blood and tissue samples were collected at the end of the study for haematology, clinical chemistry and histopathological analysis. Clinical signs, morbidity and mortality, body weights and water and food consumption were monitored during the experimental period. At the end of the experimental period sera were assessed by ELISA for total IgE and specific IgE, IgG1 and IgG2a to the test substance mCyp c 1. The concentrations of the immunoglobulins were determined from one batch of sera taken after the six-month study (n=68), one batch taken during the course of the study as an interim analysis to determine whether a certain clinical phenotype seen in the toxicological study was related to IgE/IgG values (n=19, 4 with the phenotype, 15 apparently healthy controls from all groups), and one batch of samples collected from the recovery group animals (n=7, based on serum availability).

*Statistical methods toxicity studies*

The statistical unit was an individual animal except in the food and water consumption and the urine analysis, where the statistical unit was the group of animals housed in the same cage. Continuous variables were summarized by treatment group and sex using descriptive statistics. Categorical and ordinal variables were summarized with frequencies and percentages. All statistical tests were performed as two-sided with 5 % alpha level without any adjustments. Appropriate nonparametric methods were used if the normality assumption was not met.Statistical analysis was based on two-way analysis of variance (ANOVA) for all continuous variables except for body weight and food and water consumption, where repeated measurements ANOVA was used. Effects of treatment group and sex and their interaction were introduced into the statistical model. In addition, time and its interaction with treatment group were added into the statistical model for body weight. Contrasts between treatment groups were estimated from the model separately and together for both sexes. Changes in body weight were also estimated from the model. Cochran-Mantel-Haenszel statistics was used for categorical and ordinal variables and as a nonparametric alternative for the ANOVA models.

**Results**

*Physico-chemical characterization*

In addition to the methods described in the main paper, part of the physico-chemical characterization of the molecules was based on 2D-PAGE analysis and mass spectrometry (MS). MALDI TOF MS (Figure E1) showed that the rCyp c 1 with a predicted mass of 11503 Da, comprised multiple components of higher (11604, 11659 Da) and lower (11452, 11376 Da) molecular mass. Similarly, mCyp c 1 had a predicted mass of 11328 Da and comprised two main components of 11429 and 10946 Da and multiple components resolving with m/z values between 10647 and 11826 (compared) which were less intense, suggestive of being minor components. The presence of proteins of higher or lower molecular mass may be due to truncated expression or post translational modification of the protein. This first preparation of mCyp c 1 additionally had significant contamination with *E.Coli* proteins, which was resolved during the GMP production, as can be seen on SDS-PAGE (figure 1 main paper). Additional LC/ES mass spectrometry was performed on the GMP produced mCyp c 1 (figure E3). Peak A, exhibiting a mass signal with 10,714 Da corresponds to a C-terminal truncation of 5 amino acids (aa 2-104) with a theoretical MW of 10,714 Da. Peak B, exhibiting a mass signal with 11,197 Da corresponds to the full length protein (aa 2-109) with a theoretical MW of 11,197 Da. Peak C exhibiting a mass signal with 21,909 Da corresponds to a dimer formed by a truncated (aa 2-104) and a full-length protein (aa 2-109) with a theoretical MW of 21,909 Da.

2-D PAGE of the sample (Figure E2A) showed for the rCyp c 1 a main intensely staining band of Mr 8.2 kDa together with a much weaker band of Mr 22.5 kDa. Proteomic analysis of the four marked spots (Table E1) identified these all as Cyp c 1 so the larger molecular weight material is a dimeric form (supported by its absence on SDS-PAGE under reducing conditions). For the mCyp c 1, 1D PAGE showed strong bands of Mr7.3 and 20.8 kDa with weaker bands of Mr 18.0 17.5, 8.0, 5.6 and 3.6 kDa. The 2D PAGE analysis of this sample showed multiple spots corresponding to and resolving the main bands from the 1D gel. In-gel trypsin digestion and mass spectrometry analysis of the eleven marked spots (Table E1) showed the polypeptides to comprise a mixture of parvalbumin (7 spots) and *E.coli* proteins (3 spots) and one unidentified protein. The major spots at 7.3 kDa and 20.8 kDa correspond to monomeric parvalbumin and its dimer, respectively, while a few weaker spots on the 2D PAGE gel corresponded to proteins from *E.coli*.

*Stability testing*

A stability study covering 20 months was initiated with the first batch of FAST-fish mCyp c 1 that is produced for the toxicity study. Additionally the second batch has been subjected to a similar stability study to generate more data. The appearance and color, pH, aluminium hydroxide content, total protein content and the protein in supernatant, sterility and phenol content were all complying with the predefined specifications. For the potency assay (ELISA inhibition), which is a non-validated assay, there were as yet no specifications, however, the measured values were completely stable across all measured time-points (up to 15 months) and comparable to the standard, as shown in Table E2. The inhibition ELISA was a surrogate for a true potency determination to allow stability studies of the drug product according to the Guideline on Allergen Products. In this assay the drug substance when stored frozen was used as internal control for the assay. For the drug product concentrations needed for 50% inhibition were calculated to establish stability The inhibition ELISA is currently being (pre-) validated .

*Alum-adsorbed mCyp c 1 does not display toxic effects*

As indicated in the main paper, there was a statistically significant (p<0.05) increase compared to the control in all dose groups for sIgG1, IgG2a and total IgE, and the low dose for sIgE. However, there are also differences between male and female mice (fig. E5). For sIgE, there is no statistical significant difference between the control and any of the dose groups in females, while in males only between control and low dose there is a significant difference. For IgG1, IgG2a and total IgE there is a significant difference between the control group and all dose groups in females, but not dose-dependent. In male mice the difference in IgG1 and IgG2a is significant and dose-dependent, except for the low dose IgG2a. There are no differences in total IgE for all dose groups in male mice.**Figure legends**

**Figure E1**. MALDI-TOF mass spectra(top) of rCyp c 1 (A) and mCyp c 1 (B) with the mass assignment of the peaks (bottom).

**Figure E2**. 1D and 2D SDS PAGE gels of rCyp c 1 (A) and mCyp c 1(B). Circles indicate the proteins that have been sampled and analysed using in-gel trypsin digestion and MALDI-TOF mass spectrometry (results in Table E1).

**Figure E3.** LC/ES-MS analysis of GMP m Cyp c 1. LC-UV chromatogram at 214 nm (top) with 3 major peaks (A- C) and corresponding de-convoluted mass spectra (bottom) showing the region with the highest mass signal obtained.

**Figure E4.** Results of T-cell stimulationsdepicted in the % allergen-specific cells proliferating as % of the cells proliferating in the negative control for each patient. The individual results are shown (dots) as well as the geometric mean per stimulation.

**Figure E5.** Immunoglobulin values in the mice used in repeated dose toxicity testing determined by ELISA. Open symbols: female animals; closed symbols: male animals. Statistical significant differences between dose groups are indicated in the graphs.

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