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Recombinant variants of parvalbumins, the major Fish allergens, for improved diagnosis and immunotherapy of fish allergy

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Abstract

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Type I IgE-mediated allergic reactions represent a major and increasing health problem especially in industrialized countries, where more than 25% of the population is affected by allergic diseases. These diseases are caused by *per se* harmless substances, termed allergens, which elicit in predisposed individuals a variety of different symptoms, that can involve the respiratory tract (e.g., hay fever, rhinitis, itchy nose and eyes, sneezing, coughing), the skin (e.g., urticarial, skin rashes, angioedema) or the gastrointestinal tract (e.g., nausea, cramps, vomiting, or diarrhea). The most severe allergic manifestation is anaphylaxis, a life-threatening systemic shock reaction, which impairs breathing, leads to a dramatic drop in blood pressure and affects the heart rate.

The most common inducers of anaphylactic reactions are food allergies. Ninety percent of all food allergic reactions are caused by eight major allergen sources: cow's milk, egg, wheat, soy, peanut, tree nuts, shellfish and fish. Even though fish does not represent a predominant allergen source on a world-wide basis, fish allergy can reach a prevalence of 1:1000 individuals in geographical regions with fish processing industry and high fish consumption. Interestingly, allergic symptoms to fish do not only occur after ingestion of fish, but also after inhalation of vapors generated during cooking and skin contact (reviewed in Kuehn et al. 2016).

In contrast to many other allergen sources, which contain several important allergens, allergic reactions to fish are predominantly caused by one single allergenic molecule, which is parvalbumin. Parvalbumins are evolutionary highly conserved proteins with a molecular weight of about 12 kilodalton, which have been identified as major allergens in several fish species (Elsayed et al. 1971). They belong to the family of EF-hand calcium-binding proteins and are highly abundant in the muscle tissues of all fish species. Their extraordinary resistance to heat, denaturing chemicals and proteolytic enzymes might be predisposing factors for the high sensitizing potential of parvalbumins, even as components in processed food (Elsayed et al. 1971). Fish parvalbumins show a high degree of sequence homology, which is the basis of the distinct IgE cross-reactivity between parvalbumins from different fish species and explains why many fish allergic individuals exhibit clinical symptoms upon contact with various fish species (reviewed in Kuehn et al. 2016). However, recently we and others found that there are numerous patients who react only to a single or a few fish species (Kuehn et al. 2011; Raith et al. 2014). The presence of IgE binding epitopes unique for a single parvalbumin might cause this clinical mono- or oligo-sensitivity.

Diagnosis of fish allergy is currently done by anamnesis, by *in vivo* skin prick tests with fish extracts, and by *in vitro* methods that allow determination of specific IgE antibodies to fish extracts. However, double-blind placebo-controlled food challenges (DBPCFC) are the tests with the highest accuracy in food allergy diagnosis and are still regarded as the golden standard in the diagnosis of fish allergy. Due to the potential risk of inducing dangerous reactions, these oral challenges are rarely performed outside of research settings. A major problem of the routine *in vivo* and *in vitro* methods for food allergy diagnosis is that they are carried out with poorly characterized, difficult to prepare and to standardize allergen extracts. A further problem of fish allergy diagnosis is that it is currently done with a limited number of fish extracts, which bears the risk to underdiagnose fish allergies. Even though fish allergens, especially parvalbumins, are highly cross-reactive allergens, some patients are only allergic to single fish species. Thus, there is a need for a more precise diagnostic procedure, which could be done by the use of species-specific recombinant-produced single allergens in the form of component-resolved diagnosis (CRD). We recently produced in the bacterium *Escherichia coli* a recombinant parvalbumin (rCyp c 1) from carp, a fish species frequently consumed in Austria. This recombinant allergen displayed immunological features comparable to its natural counterpart and can thus be used for component-resolved diagnosis. In the present study we aimed to produce also parvalbumins from fish species of higher global importance in a recombinant form.

The only treatment option for fish allergic individuals is currently the strict avoidance of any fish-containing food. Specific immunotherapy (SIT), the only causative treatment of type I allergy, which is based on the continuous administration of increasing doses of allergen extracts, with the aim to induce a state of allergen-specific non-responsiveness in the patient, is often used for the treatment of respiratory and venom allergies, but is not yet established for fish and other food allergies. Reasons for this are the mentioned poor quality and lack of standardization of the food extracts and the presence of several ill-defined components in these extracts. The use of single molecules instead of extracts holds promise not only for improving diagnosis of food and also fish allergy, but also for enabling specific immunotherapy of fish allergy. Another reason why SIT is not suggested for treatment of food allergies is the risk of inducing severe, life-threatening side effects. To avoid this risk, several attempts were already made with the aim to develop safer forms of immunotherapy. One of these strategies is based on the generation of hypoallergenic molecules with reduced allergenic activities which still trigger a protective Th1-response without evoking an IgE-mediated allergic reaction. One possibility to reduce the IgE binding capacity of an allergen is to modify the IgE binding epitopes of the allergens so that patients' IgE antibodies are not anymore able to bind to the allergens. We previously generated such a hypoallergenic molecule of carp (*Cyprinus carpio*) parvalbumin, Cyp c 1 (Swoboda et al. 2007). This hypoallergenic derivative is currently evaluated in an EC-funded project in a multicenter clinical trial for

its application for immunotherapy of fish allergy. To generate this hypoallergenic Cyp c 1 derivative, we previously introduced four point mutations into the two functional calcium-binding sites of Cyp c 1 (Swoboda et al. 2007). In the present study we now used the same strategy for the production of hypoallergenic parvalbumin mutants from other fish species.

With the aim to develop tools for improved diagnosis and therapy of allergies to food fish of global importance we focused on the following major fish allergens: Sal s 1 from atlantic salmon (*Salmo salar*), Thu a 1 from tuna (*Thunnus albacares*), Sco j 1 from chub mackerel (*Scomber japonicus*), Clu h 1 from herring (*Clupea harengus*), and Gad c 1 from codfish (*Gadus morhua*), all parvalbumins from fish species often consumed but distantly related to carp (Swoboda et al. 2013). Synthetic genes codon-optimized for expression in *Escherichia coli* coding for the wildtype forms and for mutated variants of these parvalbumins were synthesized by the company GenScript. The design of the mutants was based on the knowledge that exchange of particular amino acids at positions 1 and 3 of the two calcium-binding domains had led to a hypoallergenic molecule which did not show binding of patients' IgE antibodies in case of carp parvalbumin (Swoboda et al. 2007). These particular amino acids had been targeted because they are known to be involved in the formation of the calcium-binding pockets and thus for the coordination of the calcium ions in all EF-hand calcium-binding proteins (Zhou Y. et al. 2009). The mutants all carried the same 4 point mutations in the two calcium-binding regions as the carp parvalbumin mutant, leading to the exchange of the aspartic acids (D) at the first and third positions of the two functional calcium-binding domains to alanines (A). The wildtype and mutant synthetic genes were cloned into the *Nde*I and *EcoR*I restriction sites of the pET17b vector for further expression in the *Escherichia coli* strain BL21 (DE3). In addition, all expression constructs carried a C-terminal histidine-tag for easier purification of the recombinant parvalbumin proteins by nickel NTA affinity chromatography. Recombinant proteins were then characterized by sodium dodecylsulfate polyacrylamid gel electrophoresis (SDS-PAGE) and in immunoblots using antibodies directed against the histidine-tag or against different parvalbumins. Coomassie-stained protein gels and immunoblots with the anti-histidine antibodies showed that all wildtype and mutated proteins could be successfully purified from the bacterial lysates and showed similar migration behavior. Further characterization using different anti-parvalbumin antibodies showed that wildtype and mutant variants were recognized by the antibodies to a different extent. In general, the majority of the anti-parvalbumin antibodies displayed stronger reactivity to the different wildtype parvalbumins than to the mutants.

As a next step, IgE reactivities of the wildtype and mutant proteins were analyzed in immunoblots using a pool of sera from fish allergic patients with a known sensitization to parvalbumin. For this, wildtype and mutant proteins were separated by a denaturing SDS-PAGE, blotted onto nitrocellulose membrane and incubated with the pooled patients' sera. A HRP-labeled anti-human IgE antibody and a SuperSignal West Pico Chemiluminescent Substrate were used to detect binding of parvalbumin-specific IgEs. These IgE immunoblots showed that the wildtype parvalbumins of all tested fish species were recognized by patients' IgE antibodies, whereas the mutant variants did not bind any IgEs. To analyze the molecules' IgE reactivity also under native conditions and to evaluate patient-specific differences, we tested IgE reactivity of individual patients' sera to the wildtype and mutated parvalbumins in enzyme-linked immunosorbent assays (ELISA). For this, we coated ELISA plates with the different parvalbumins from salmon, tuna, chub mackerel and codfish or, for control purposes, also from carp and incubated them with the individual patients' sera. Anti-IgE antibodies linked with alkaline phosphatase were used to detect bound patients' IgEs and substrate was added to visualize the reaction. ELISA data revealed that all patients' sera showed IgE reactivity to the wildtype variants of parvalbumin. However, many sera displayed IgE reactivity of varying intensity to the wildtype proteins and some sera displayed a very selective IgE recognition of parvalbumins from certain fish species. These results indicated that also among our patients some patients are preferentially sensitized to specific fish species and that our recombinant parvalbumins would allow to identify this selective reactivity.

It was important to see that none of the mutated variants were recognized by IgE antibodies from fish allergic individuals. These data demonstrated that mutation of exactly the same calcium-coordinating amino acids as in the carp parvalbumin Cyp c 1 also resulted in hypoallergenic molecules in case of Sal s 1, Thu a 1, Sco j 1 and Gad c 1, as shown by the strongly reduced IgE reactivity under denaturing and also under native conditions. Comparable to the Cyp c 1 mutant, the other mutated parvalbumins also have the potential to be used for immunotherapy of patients with a selective IgE reactivity to certain fish species.

We thus produced recombinant fish allergens and fish allergen mutants, which have the potential to be used for improved diagnosis and immunotherapy of fish allergies. To further characterize the recombinant wildtype and mutant molecules, their structure will in future be analyzed by circular dichroism analysis under native and denaturing conditions and their calcium-binding capacity will be evaluated by inductively coupled plasma mass spectrometry (ICP-MS).

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