Abstract of Doctoral Dissertation

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Title of Doctoral Dissertation

Structural and Stability Analysis of GRP Family Allergens Pru p 7 and Cry j 7, Which Cause Pollen and Food Allergy Syndrome

（花粉食物アレルギー症候群の原因となる GRP ファミリーアレルゲン
Pru p 7 および Cry j 7 の構造および安定性の解析）

Pollinosis prevalence is on the increase worldwide, and recent reports show that the pollen allergy is going to be a greater problem than before. Thus, it is crucial to comprehend all the structural features of allergenic proteins. Recently, a small gibberellin-regulated proteins (GRP) isolated from Japanese cedar (*Cryptmeria japonica*), Cry j 7, was identified as pollen allergen can make cross-reactivity with food allergens cause pollen-food allergy syndrome (PFAS). GRPs, also known as the Snakin/Gibberellic-acid-stimulated Arabidopsis (GASA) protein family, exhibit antimicrobial properties, and their amino-acid sequence contains 12 cysteine residues forming 6 disulfide bridges in conserved positions, conferring thermostability and proteolytic resistance. The GRP isolated from peach fruit is the first GRP to be identified as an allergen and was named Pru p 7. To date, allergenic homologs found in other plant-food were described, named Pru m 7 from Japanese apricot, Cit s 7 from orange, Pun g 7 from pomegranate, and Cap a 7 from bell pepper. Subsequent extensive investigations have confirmed their cross-reactivity with pollen GRPs reported in Cupressaceae family, such as Cup s 7 from Europe cypress (*Cupressus sempervirens*) and Jun a 7 from Mountain cedar (*Juniperus ashei*). At present, the actual pathogenic mechanism of food allergies linked to the GRP family is less detailed, and studies on reactions to pollen GRP are also rare. In one Japanese cohort, the incidence of food allergy mediated by GRPs was 65% patients reacting to peach. Another study of a cohort of young Japanese patients allergic to Japanese cedar pollen and to fruit showed that 46% are sensitized to Cry j 7. Further studies are necessary to explore the characteristics of GRP allergens, as well as the cross-reactivity between them to help us understand the allergenicity mechanisms of PFAS.

In this study, the methylotrophic yeast *Pichia. pastoris* was chosen due to its superior capacity to undergo post-translational modifications, such as the intracellular creation of disulfide bridges and the extracellular secretion of folded proteins. Recombinant Cry j 7 and Pru p 7 were successfully overexpressed using *P. pastris* in a high cell-density fermentation culture and purified with a cation exchange column followed by reverse phase-HPLC. The yield estimated from the absorbance at 280 nm for purified Cry j 7 was 20 mg from 1 L fermentation culture, while Pru p 7 produced 45 mg of protein from that. The characteristics of Cry j 7 and Pru p 7 were identified by MALDI-TOF MS, CD, and ^{1}H-NMR experiments, confirming the correct native conformation of Cry j 7 as well as Pru p 7.

In NMR research, the solution structures of Cry j 7 and Pru p 7 were determined. This was the first report of the NMR structural characterization of the Japanese cedar pollen allergen Cry j 7, and it also improved the intact three-dimensional structure of the peach allergen Pru p 7. Compared with the previously published 3D-

structural model of Pru p 7 using a natural protein sample, we performed NMR experiments on stable isotope-labeled recombinant proteins to obtain more accurate 3D-structure information. All backbone resonances of Pru p 7 and Cry j 7 were completely assigned, and the proper connection of 6 disulfide linkages among 12 cysteine residues was further determined. The stable bond pairs were as follows: Cys5-Cys30, Cys9-Cys26, Cys13-Cys22, Cys29-Cys62, Cys33-Cys49 and Cys35-Cys47. Besides, the Cry j 7 and Pru p 7 displayed high similarity in mainly helical structures in their N-terminal region while appearing differences in the C-terminal flexible region according to NMR results. In ^{1}H-^{15}N NOE, the significant difference in the P38/S38-C47 region revealed the high mobility of Pru p 7 and low mobility of Cry j 7, which corresponded to the molecular dynamic simulation results. In these flexible regions composed of loops and a short α-helical structure, there are differences in charge residues and hydrogen bonds, and these might affect the mobility, although the impact of significant differences in the flexibility of this region on immunogenicity to allergens necessitates further exploration. At the same time, these results are consistent with the conclusions of thermal denaturation experiments using CD and ^{1}H-NMR, clearly supporting the structural characteristics of Cry j 7, which is more stable than Pru p 7 in terms of physicochemical aspects. In particular, the finding that heating under alkaline conditions promotes irreversible structural denaturation of Pru p 7 compared to acidic conditions is of great significance when considering the removal of allergenicity by processing.

Sensitizations occur via cutaneous or airway exposure of allergens, or when food allergens enter the human gastrointestinal tract to induce the immune response. In this study, we also evaluated the resistance to gastrointestinal digestion and endosomal degradation associated with sensitization. Interestingly, the degradation by enzymes in the digestive tract shows the opposite trend to thermal denaturation, and Pru p 7 was more stable. Therefore, the difference in stability to these digestive enzymes may be attributed to the specificity of the enzyme rather than structural stability. Endosomal digestion is involved in the establishment of sensitization through the production of allergen-derived peptides presented by MHC II. The results pointed that Cry j 7 and Pru p 7 were easily digested by cathepsin S enzyme in the reduced state. On the other hand, the structures of Cry j 7 and Pru p 7 in their oxidized states showed high stability. This high degradation efficiency in reduced state may induce a Th2 response by efficiently producing allergen-derived peptides and binding them to MHC II, and promoting the production of specific IgE by B cells.

In conclusion, analysis of the three-dimensional structure and thermal stability using CD and NMR in this study revealed that Cry j 7 is more stable than Pru p 7. The differences in the mobility of the characteristic loop regions of the two proteins were clarified, and it was suggested that these loop regions not only contribute to stability but may also be epitope sites. In contrast, Pru p 7 had higher resistance to degradation by digestive enzymes, and analysis of the degradation fragments suggested that the difference in the primary sequence was contributing to the resistance. In addition, although the resistance of endosomal enzymes involved in the establishment of sensitization was greatly reduced by the reduction of disulfide bonds in both proteins, the fact that the loop region in the native structure was cleaved to some extent preferentially suggests that the remaining structure after reduction may also be related to the resistance. A detailed comparison of the structure and stability of the GRP family derived from pollen and fruit in this study will be useful as basic information for elucidating the mechanisms of sensitization and the development of allergies in PFAS in the future.