



Rice seed ER-derived protein body as an efficient delivery vehicle for oral tolerogenic peptides

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ABSTRACT

Mucosal delivery of peptide/protein therapeutics via the oral route is a desirable strategy in human immunotherapy. A key step for enhancing the bioavailability of orally administered therapeutics is to protect them from enzymatic digestion in the gastrointestinal tract. Here, we generated transgenic rice seeds accumulating allergen-derived T cell epitopes, a model tolerogen for the control of pollen allergy, in either ER-derived protein body-I (PB-I) or protein storage vacuole protein body-II (PB-II). Compared with PB-II-localized or chemically synthesized forms, PB-I-localized T cell epitopes showed higher resistance to enzymatic digestion in simulated gastric fluid. Moreover, the dose of T cell epitope required for suppression of allergen-specific IgE in mice was about 20-fold lower when fed in PB-I localized form than when unprotected. These findings demonstrate the potential of bioencapsulation in PB-I for broad applications as a viable strategy to achieve efficient mucosal delivery of oral peptide/protein therapeutics.

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1. Introduction

Oral administration of peptide/protein therapeutics offers a convenient, inexpensive, and patient-friendly strategy for modulating immune responses. However, the problem of low bioavailability of orally administered therapeutics, mainly due to enzymatic digestion in the gastrointestinal tract, remains to be solved before this approach becomes practical [10,11]. In recent studies, recombinant protein products expressed in plant tissues have been shown to be protected from proteolytic digestion in simulated gastric fluids, indicating their potential as a novel platform for the production and bioencapsulation of agents for efficient mucosal delivery of therapeutics [1,7]. Here, we evaluated the biochemical and immunological properties of rice seed as a mucosal delivery vehicle for oral peptide/protein therapeutics.

In rice seed, more than 80% of the total seed proteins are deposited as storage proteins in protein bodies as protein storage organelles [12]. They are thereby protected by membrane boundaries from proteases and desiccation during seed maturation until the protein is required as a nitrogen source for the germinating

seedling [6]. Thus, the protein bodies have long been considered to be suitable sites for deposition and storage of large amounts of recombinant products. Rice seed contains two types of protein bodies; the ER-derived protein body called PB-I, which accumulates several types of prolamin, and the protein storage vacuole protein body designated PB-II that comprises glutelins and globulin [18]. Compared with glutelins in PB-II, prolamins in PB-I are highly resistant to gastrointestinal digestion *in vivo* and *in vitro* [8,15], suggesting that PB-I may be the better candidate for protection of products from digestion. On the other hand, a significant amount of PB-I containing prolamins are excreted and can be detected in human feces [8,20], raising questions about the efficiency of PB-I for mucosal delivery of products. Therefore, further studies are required to evaluate the biochemical and immunological significance of PB-I and PB-II as mucosal delivery vehicles.

Oral administration of allergen-derived T cell epitopes is known to induce immunological tolerance useful for the control of allergy [2,4,13]. In a previous study, a synthetic peptide designated 3Crp, composed of three T cell epitopes derived from the Japanese cedar pollen allergens Cry j 1 and Cry j 2, was used as a model oral tolerogen in BALB/c mice [22]. In the present study, our efforts were initially focused on the development of three forms of 3Crp, (i) chemically synthesized, (ii) PB-I-localized, and (iii) PB-II-localized 3Crp. To engineer 3Crp accumulation in PB-I, 3Crp was expressed in rice as a fusion protein with a synthetic 7Crp peptide [16], composed of human T cell epitopes of Cry j 1 and Cry j 2, because 7Crp is known to predominantly accumulate in PB-I by interaction with cysteine-rich prolamins due to the presence of free cysteine residue

Abbreviations: PB-I, ER-derived protein body-I; PB-II, protein storage vacuole protein body-II; GLU, rice glutelin acidic subunit.

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[17] and the 3Crp peptide itself cannot be accumulated in seed due to the short length (42 amino acids) [14]. To achieve PB-II-specific accumulation of 3Crp, it was expressed as a part of glutelin by inserting into the C-terminal highly variable region in the acidic subunit of rice glutelin precursor. Here, we examined their accumulation sites in rice seed, in vitro digestibility, and oral tolerogenicity in a mouse model of Japanese cedar pollen allergy.

2. Materials and methods

2.1. Plasmid construction and rice transformation

The codon-optimized 3Crp gene was amplified by PCR [14] and cloned into the Nco I site of the pGluBpro sig/7CrpKDEL plasmid [16], producing a fusion protein between 3Crp and 7Crp (designated 3Crp–7Crp, Fig. 1). For PB-II-specific accumulation of 3Crp, an expression plasmid coding for a fusion protein between 3Crp and the rice glutelin acidic subunit (designated GLU–3Crp) was constructed as described previously (Fig. 1) [14]. Each expression plasmid was introduced into the rice genome (*Oryza sativa* L. cv Kitaake) via *Agrobacterium tumefaciens*-mediated transformation [16].

2.2. SDS-PAGE and Western blot analysis

More than 30 independent rice lines were generated for studies on 3Crp accumulation in mature seed, undertaken by SDS-PAGE and Western blotting using rabbit antibodies raised against either 3Crp [14] or 7Crp [16]. To prepare a standard for 3Crp quantification, 3Crp–7Crp or GLU–3Crp proteins were expressed as 6×His-tagged proteins in *E. coli* and purified by affinity chromatography. Amounts of 3Crp accumulated in the seeds were read off against the standard curve generated by densitometric scanning of Western blots of the purified standard.

2.3. Immunoelectron microscopy

To determine the intracellular localization of 3Crp–7Crp and GLU–3Crp, sections of the endosperm of developing seed at 20 days after flowering (20 DAF) were prepared as described previously [17]. Either anti-3Crp antibodies (1/10 dilution) or anti-7Crp antibodies (1/10 dilution) were used as primary antibodies, and goat anti-rabbit IgG conjugated to 25 nm gold colloidal particles (1/200 dilution) was used as a secondary antibody. Grids were then stained with 2% (w/v) uranyl acetate and lead citrate for electron microscopy.

2.4. In vitro digestion by pepsin and pancreatin

In vitro digestibility properties were examined as described previously [15]. Briefly, 3Crp peptide was synthesized by standard F-moc methodology and purified by HPLC; 36 µg of 3Crp peptide was then dissolved in 360 µl of 100 mM sodium acetate (pH 1.7) and 30 mM sodium chloride with 0.32% (w/v) pepsin (Sigma), or 50 mM potassium dihydrogen phosphate (pH 7.5) with 1% (w/v) pancreatin (Nacalai Tesque, Japan). 40 mg of fine powder of 3Crp–7Crp seeds containing 36 µg of 3Crp–7Crp or a mixture of 20.5 mg of GLU–3Crp seed powder containing 36 µg of GLU–3Crp with 19.5 mg of non-transgenic rice seed powder was dissolved in the reaction mixtures. These digestion reactions were incubated at 37 °C for up to 18 h as described in Fig. 3 and terminated with the addition of protein extraction solution [15]. Thereafter, total protein in each reaction mixture was separated by SDS-PAGE (3Crp–7Crp and GLU–3Crp seed) or by Tricine-SDS-PAGE (chemically synthesized 3Crp peptide) [9], and assayed by Western blotting.

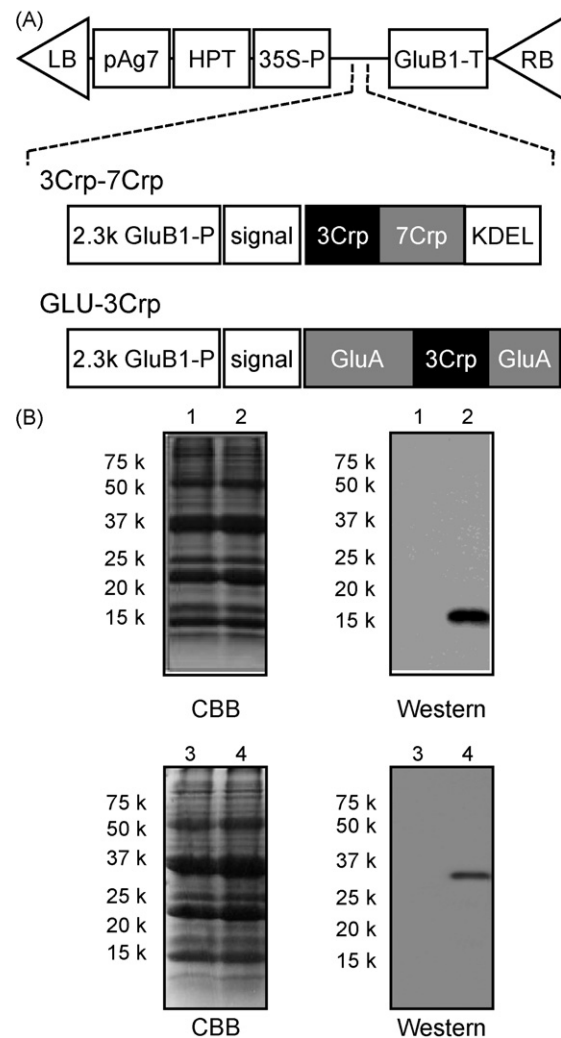


Fig. 1. Expression of 3Crp–7Crp and GLU–3Crp in rice seed. (A) Constructs for the expression of 3Crp–7Crp and GLU–3Crp in rice seed. DNAs coding for 3Crp–7Crp and GLU–3Crp [14] were linked to the rice seed storage protein glutelin *GluB1* promoter. Hygromycin phosphotransferase (HPT) was used for the selection of transgenic rice plants. *GluA*, rice glutelin *GluA*; *GluB1-P*, rice glutelin *GluB1* promoter; *GluB1-T*, *GluB1* terminator; KDEL, tetrapeptide ER retention signal; 35S-P, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase; pAg7, agropine synthase polyadenylation signal sequence; LB, left border; RB, right border. (B) Detection of 3Crp–7Crp and GLU–3Crp accumulated in rice seed. Total protein from rice seed was separated by SDS-PAGE and subjected to CBB staining and Western blot analysis. Lane 1, non-transgenic rice seed; lane 2, 3Crp–7Crp seed, lane 3, non-transgenic rice seed; lane 4, GLU–3Crp seed. Accumulation of GLU–3Crp in rice seed was previously examined [14].

2.5. Oral tolerance induction and sensitization of mice

To compare oral tolerogenicity of the three formulations of 3Crp, 6-week old male BALB/c mice (CLEA Japan) were given chemically synthesized 3Crp, or 200 mg fine powder of 3Crp–7Crp or GLU–3Crp seed orally in 1.0 ml PBS once a day for 10 days ($n=4$ or 5 in each group). Different doses of 3Crp as described in the text were prepared by mixing non-transgenic rice seed with 3Crp–7Crp or GLU–3Crp seed. After tolerogen feeding, mice were challenged intraperitoneally with 0.1 mg of total protein extract of Japanese cedar pollen (Cosmo Bio, Japan) adsorbed on 5 mg of aluminum hydroxide (Cosmo Bio) containing recombinant mouse IL-4 (R&D Systems) at 0.1 µg per mouse to maximize the induction of allergen-specific IgE responses [13]. One week later, mice were boosted with 0.1 mg of total protein extract of Japanese cedar pollen adsorbed on 5 mg of aluminum hydroxide. Mouse experi-

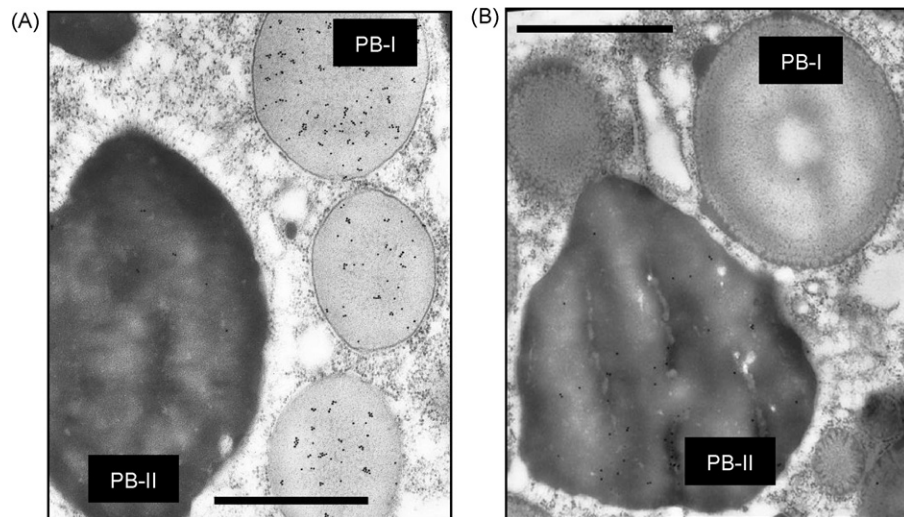


Fig. 2. Immunoelectron micrographs of 3Crp-7Crp (A) and GLU-3Crp (B) accumulated in developing rice seed at 20 DAF. Rabbit anti-7Crp (A) and anti-3Crp (B) antibodies were used as primary antibodies, and goat anti-rabbit IgG conjugated to 25 nm gold colloidal particles was used as the secondary antibody. Bar = 1 μ m.

mental protocols were approved by the Experimental Animal Care and Use Committee of the Tokyo Metropolitan Institute of Medical Science.

2.6. ELISA

Mice were bled to measure allergen-specific serum IgE titers by ELISA as described previously [13]. Briefly, ELISA plates were coated with 2 μ g/ml of anti-mouse IgE (Southern Biotechnology), and serial dilutions of serum were applied to the plates. Biotinylated pollen extract was used as the secondary reagent. Streptavidin–horseradish peroxidase conjugate (Pierce) was then added to the plates, and the reaction was developed with peroxidase substrate solution (Moss). The last serum dilution yielding an OD450 value of 0.1 over the background was recorded as the endpoint titer.

3. Results

3.1. Localized accumulation of 3Crp in PB-I or PB-II in transgenic rice seed

Constructs coding for 3Crp-7Crp or GLU-3Crp were expressed in rice endosperm under the control of the 2.3 kb glutelin *GluB1* promoter (Fig. 1A). A 3Crp-7Crp product migrated on polyacrylamide gels with approximately the expected molecular mass (16.9 kDa). An amount estimated at 18 μ g/20 mg seed was present in homozygous T3 seed (Fig. 1B). A GLU-3Crp signal had been previously documented as a band with the expected molecular mass of 34 kDa at approximately 35 μ g per 20 mg seed [14].

The intracellular localization of 3Crp-7Crp and GLU-3Crp proteins in the endosperm cells of transgenic rice was investigated by immunoelectron microscopy. The 3Crp-7Crp signal was detected predominantly in PB-I, while GLU-3Crp was localized in PB-II (Fig. 2). No signal was detected in endosperm cells of non-transgenic rice seed (data not shown).

3.2. In vitro digestibility of 3Crp

Chemically synthesized 3Crp and PB-II-localized GLU-3Crp were found to be completely digested by pepsin within 2 and 30 min, respectively (Fig. 3A). In contrast, PB-I-localized 3Crp-7Crp was highly resistant to pepsin even after 120 min and was com-

pletely digested after 14 h of digestion (Fig. 3A). In the case of pancreatin, chemically synthesized 3Crp was completely digested within 2 min, whereas PB-II- and PB-I-localized 3Crp remained detectable at 30 min and 18 h, respectively (Fig. 3B). These results indicate that PB-I-deposited form of 3Crp was the most effectively protected from enzymatic digestion.

3.3. Oral tolerogenicity of 3Crp in a mouse model of allergy

Before comparing oral tolerogenicity, we undertook pilot studies to investigate the effects of the 3Crp feeding period on tolerance induction in our experimental mouse model. Daily oral feeding of GLU-3Crp seed containing 1.5 μ g of 3Crp for 25 or 50 days did not suppress allergen-specific IgE responses, whereas a feeding period of 5 days using seed containing 15 μ g of 3Crp was sufficient to do so (Fig. 4A). In the groups of mice fed chemically synthesized 3Crp peptide, more than 100 μ g of 3Crp peptide was found to be required for suppression of allergen-specific IgE responses (Fig. 4B). In contrast, in the groups of mice fed PB-I-localized 3Crp-7Crp, as little as 5 μ g of 3Crp (a 20-fold lower doses than required for the chemically synthesized 3Crp peptide) was sufficient to confer IgE suppression (Fig. 4C), while a comparable amount (6 μ g of 3Crp) of PB-II-localized GLU-3Crp did not give rise to IgE responses (Fig. 4A) [14]. These results indicate that oral delivery of 3Crp in PB-I-localized form significantly enhanced its efficacy for suppression of allergen-specific IgE responses.

4. Discussion

PB-I-localized 3Crp exhibited higher resistance to enzymatic digestion and more effectively induced IgE suppression than either the chemically synthesized or PB-II-localized forms. Better protection of PB-I formation against proteolytic degradation than the PB-II formation may be related to high polymerization by Cys-rich prolamins through disulfide bond formation. Therefore, bioencapsulation of 3Crp as a tolerogen in PB-I provides an efficient delivery system to gut-associated lymphoid tissue (GALT), resulting in facilitation of oral administration and contributing to overcoming the low efficacy of oral administration of native peptide/protein itself. Furthermore, direct administration of transgenic rice grains containing tolerogen offers an inexpensive means to deliver high doses of tolerogen, because higher amounts of tolerogen can be accumu-

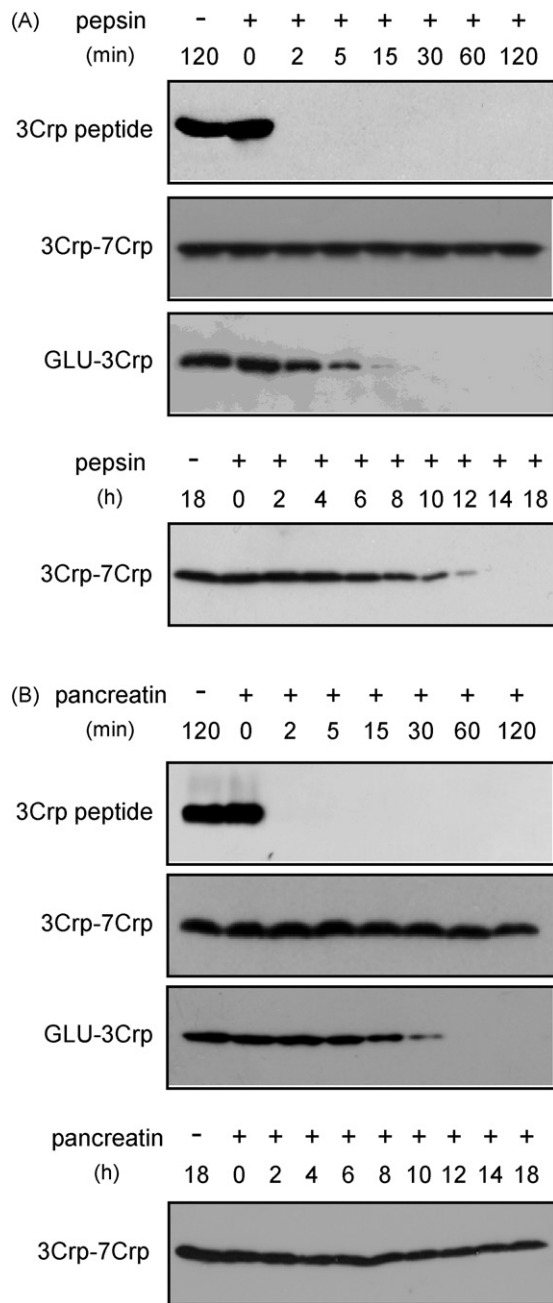


Fig. 3. In vitro digestibility of the three forms of 3Crp. Reaction mixtures containing pepsin (A) or pancreatin (B) were incubated at 37 °C for up to 18 h. Total protein in each reaction mixture was then extracted and examined by Western blotting.

lated in rice grains and they are easily propagated by cultivation without specific facility dependent on the demand.

In this study, there remains a concern that glutelin and 7Crp fused to 3Crp may contribute to modulating the tolerogenicity and susceptibility of 3Crp peptide. A previous study showed that feeding of non-transgenic rice seed containing glutelin does not induce oral tolerance against cedar pollen allergens in mice [13], indicating that rice glutelin does not affect the tolerance induction. The 7Crp peptide contains the sequence of Cry j 2 p77–83, which is included in the subdominant T cell epitope region (Cry j 2 p70–83) recognized by BALB/c mice [22]. However, a recent study showed that feeding of synthetic Cry j 2 p70–83 peptide to BALB/c mice does not suppress the response against Cry j 2 [3], suggesting that the potential contribution of Cry j 2 p77–83 containing 7Crp peptide to the

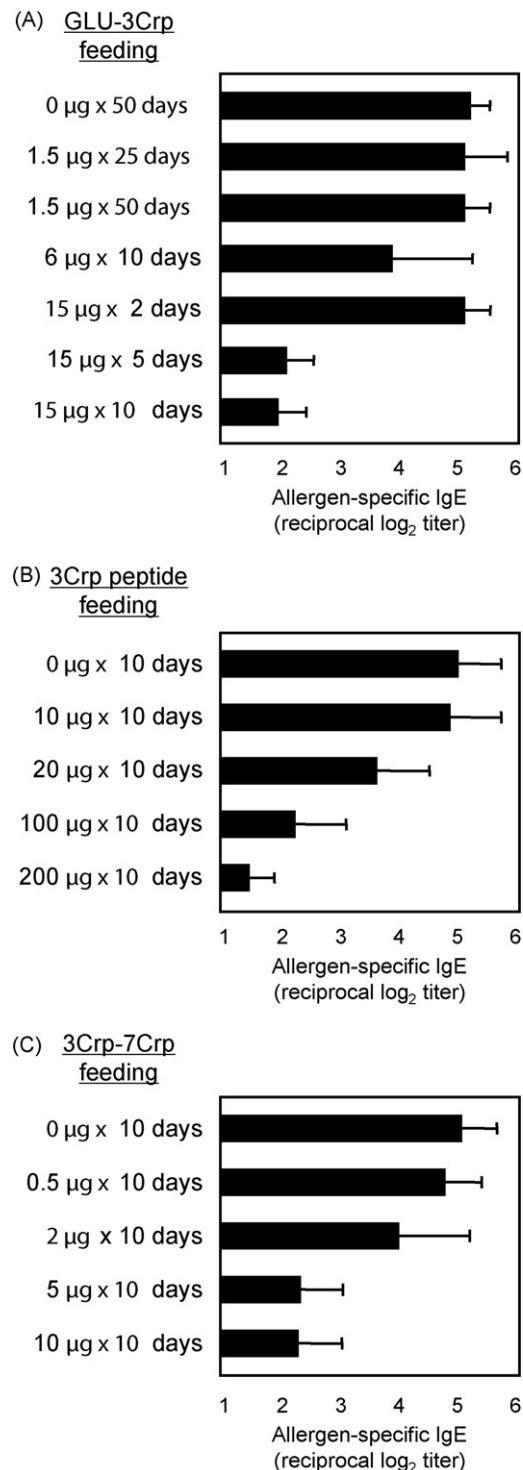


Fig. 4. Suppression of allergen-specific IgE responses after oral administration of the three forms of 3Crp. Mice in groups of 4–5 were given either GLU-3Crp seed (A), chemically synthesized 3Crp peptide (B) or 3Crp-7Crp seed (C) orally and then intraperitoneally challenged with total protein extract of Japanese cedar pollen. Allergen-specific serum IgE titers were then determined by ELISA.

tolerance induction is excluded. In addition, both glutelin and 7Crp accumulated in rice seed are easily digested in a similar minute by pepsin in vitro [15], therefore, although other factors may influence the tolerogenicity and susceptibility of 3Crp, it would be reasonable to conclude that bioencapsulation of 3Crp in PBs contributes to protection from digestion and enhancement of bioavailability.

To enhance the efficacy of plant-based therapeutics, a major strategy has been to increase the levels of accumulation of antigens or tolerogens in plant cells. Improvement of several key factors, including the use of tissue-specific strong promoters, translational/post-translational control, and callus-specific selection of transformed plants as well as intracellular compartment presented here, has been applied to yield higher levels of a number of therapeutics in plants [5,19,21]. Furthermore, efficacy of therapeutic products accumulated in rice seed has been demonstrated in animal models, proving the clinical potential of rice-based production strategies for applications in human therapy [7,17,19]. Here, our findings indicate that the nature of the subcellular localization in plant cells in which the agent accumulates is an important factor affecting the digestibility and immunological efficacy of plant-based oral therapeutics. In this regard, this study exemplifies a novel concept and technology for improving the bioavailability of mucosal therapeutics, thus contributing to increasing the possibility of clinically applicable plant-based oral therapeutics.

Intended for human use, the 7Crp hybrid T cell epitope has been specifically and highly expressed in rice seed under the control of the endosperm-specific glutelin promoter, and was found to accumulate predominantly in PB-I [16,17]. Therefore, a PB-I-based strategy using 7Crp seed is now adaptable for human allergy therapy. However, it is important to note that oral administration of 3Crp at lower than threshold amounts did not suppress IgE responses even if the same total amounts of 3Crp (75 µg) as that required for induction of tolerance was administered using the GLU-3Crp seeds over a long period (50 days) (Fig. 4A). This evidence suggests that the oral tolerance may operate through different mechanisms, since administration of long-term low-dose oral antigen is known to preferentially induce regulatory T cells essential for long-lasting protection (Fig. 4A). Thus, prior to clinical application, the dose and duration of 7Crp seed administration for tolerance induction requires careful investigation.

5. Conclusion

Rice seed PB-I-based bioencapsulation and mucosal delivery strategies significantly improve the bioavailability of orally administered peptide/protein therapeutics. Thus, PB-I offers a novel practical platform for overcoming the low efficacy of oral therapeutics.

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