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Establishment of monoclonal antibodies of BW10kDa distinguish it from Fag e 2 related to anaphylaxis

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ABSTRACT

BW10kDa, which is a buckwheat (BW) allergen, belongs to the 2S-albumin protein family, akin to Fag e 2. Detailed analyses of BW10kDa were lacking until this study. Herein, we conducted these analyses using monoclonal antibodies (mAbs) to recombinant BW10kDa (rBW10kDa). We successfully generated anti-rBW10kDa mAbs capable of distinguishing between Fag e 2 and BW10kDa. These mAbs were categorised into two types (type 1 and type 2) based on their reactivity to BW plant seed extracts in western blot analyses. Type 1 mAbs revealed two bands (15 kDa and 10 kDa), while type 2 mAbs showed a single band (15 kDa). Spot analyses using these mAbs confirmed that type 1 mAbs recognised epitopes near the C-terminal region, with the 10 kDa band representing the C-terminal subunit cleaved by protease. The mAbs targeting rBW10kDa enabled to assess the concentration of BW10kDa in wild type and also in diagnostic buckwheat extracts.

1. Introduction

Buckwheat (*Fagopyrum esculentum)* is an annual herbaceous plant of the genus Fagopyrum and family Polygonaceae, that is cultivated for its grain-like seeds. It is not only consumed in Japan and China, where it originated, but also in various other countries around the world, as the staple, in dishes such as cold noodles in Korea, galette in France, and pizzoccheri in Italy. Buckwheat is a rich source of protein and has attracted attention as a health food, but on the other hand, it is, also a common cause of food allergies; serious allergic reactions, including anaphylactic shock, affecting all age groups have been reported (Smith, 1990). Some of allergens in buckwheat are also heat-resistant, and allergies can develop through consuming extracts obtained after boiling buckwheat. Among the buckwheat allergens, anaphylactic reactions have been reported with Fag e 2 (Koyano et al., 2006), Fag e 3(Cho et al., 2007), and BW10kDa (Matsumoto et al., 2004).

Fag e 2 belongs to the 2S albumin protein family and seems to be the causative allergen of anaphylactic reactions (Tanaka et al., 2002). Multiple allergens have been reported to belong to the 2S albumin protein family (Mills et al., 2004; Breiteneder and Radauer, 2004). Members of the 2S albumin protein family are predicted to have a compact three-dimensional structure with four disulfide bonds formed by eight well-conserved cysteine (Cys) residues and have highly similar α-helical structure.

Fag e 3 is a protein thought to belong to the cupin superfamily, which includes multiple allergens. Although Fag e 3 is pepsin-sensitive (Tanaka et al., 2002), Fag e 3-specific IgE has been reported that could predict oral food challenge (OFC) results and OFC-induced anaphylaxis (Yanagida et al., 2018).

BW10kDa is an allergen belonging to the 2S albumin protein family, which has also been reported to be BW8kDa (Matsumoto et al., 2004), and has 54.1 % identity with Fag e 2 (Koyano et al., 2006). It is reported that BW10kDa belongs to the 2S albumin protein family and is one of the major buckwheat allergens that reacts with the sera of 57 % of buckwheat allergic patients (Matsumoto et al., 2004). However, detailed analyses of BW10kDa, including its origin, have not yet been conducted.

Recent studies by Yasui et al. (2016) identified 2S-albumin family genes (g14, g11, g28, g13) in common buckwheat, while Katsube-Tanaka and Monshi (2022) reported the existence and characterisation of 2S-albumin family allergic proteins. These reports suggest that Fag e 2 is encoded by the g14 gene, whereas BW10kDa is encoded by the g11 and g28 genes, indicating differences in their accumulation.

Our study aimed to explore the potential differentiation between Fag e 2 and BW10kDa using monoclonal and polyclonal antibodies

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developed by our team. Additionally, we sought to conduct detailed analyses of BW10kDa to elucidate its role, alongside Fag e 2, in anaphylactic reactions triggered by buckwheat consumption.

2. Materials and methods

2.1. Preparation of the recombinant BW10kDa (rBW10kDa) protein

Based on the previously reported amino acid sequence of the 10-kDa allergen (BW10kDa, 133 aa; Swiss Prot #Q8W3Y9), the deduced amino acid sequences (114 aa), excluding the leader sequences (1–19 aa) (Fig. 1), were cloned into the Novagen-transcription vector, pET21a. This vector harbours a C-terminal His-Tag sequence (Merck kGaA, Darmstadt, Germany). In details, the cDNA encoding amino acid residues 20–133 was amplified using forward (5′-GGGGGATCCGACAGCCAGATGAGGTCGAAATGC-3′; underline denotes introduced *Bam*HI site) and reverse (5′-CCCGAATTCTTACTCGTAATCCCTAGTTCCGAT-3′; underline denotes introduced *Eco*RI site) primers and ligated between the *Bam*HI and *Eco*RI sites of pET21a vector and cloned. The expression vector pET21a-BW10kDa was transfected into Novagen-competent *E. coli* BL21 (DE3) (Merck kGaA, Darmstadt, Germany) to obtain recombinant BW10kDa (rBW10kDa). The obtained *E. coli* strains containing the recombinant protein were cultured in TB (Terrific Broth, Thermo Fisher Scientific Inc., Waltham, MA, USA) medium containing 0.5 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside), and cultured for 18 h at 20 °C. The cells were then collecteted by centrifugation, suspended in PBS and disrupted by sonication. After that, the insoluble fraction (inclusion body) was collected by centrifugation and suspended in PBS containing 8 M Urea and 5 mM imidazole for solubilizing the inclusion body and rewinding the denatured protein. Then soluble fraction was applied to HisTrap HP column (Cytiva, Uppsala, Sweden) and rBW10kDa was then eluted from the HisTrap HP column with 100–200 mM imidazole, with a yield of 95 mg/200 mL. SDS-polyacrylamide gel electrophoresis (PAGE) confirmed the purification of the His-tag attached full-length rBW10kDa by Coomassie brilliant blue (CBB) staining and study of western blotting using Penta His antibody (Qiagen, Venlo, Netherland) and Amersham ECL Prime Western blotting reagent (Cytiva, Uppsala, Sweden).

2.2. Preparation of antibodies directed against r-BW10kDa

2.2.1. Preparation of polyclonal antibodies (pAbs)

After two rabbits were immunized by subcutaneous injection of r-BW10kDa (250 μg/dose) a total of 5 times (with Freund's complete adjuvant (FCA) the first time and Freund's incomplete adjuvant (FIA) the second time onwards), whole blood was collected from the rabbits and the serum fraction containing pAbs directed against rBW10kDa was separated.

2.2.2. Preparation of monoclonal antibodies (mAbs)

After mice were immunized by subcutaneous injection of rBW10kDa (100 μg/dose) a total of 5 times (with FCA the first time, and FIA the second time onwards), hybridomas were prepared from the mouse spleen cells and mAbs directed against rBW10kDa were obtained from the culture supernatants of five types of cloned cells (1E7, 7D6, 8C1, 2F9, 16F9).

2.3. Preparation of recombinant Fag e 2(rFag e 2) and antibodies directed against rFag e 2

The expression and purification of rFag e 2 in *E. coli* were performed as described in a previous report (Koyano et al., 2006). Polyclonal antibodies directed against purified rFag e 2 (rFag e 2-pAbs) were obtained from the whole blood of rabbits collected after the animals had been immunized by subcutaneous injection of rFag e 2 (250 μg/dose). Another antibody directed against the Fag e 2 peptide sequence ^{121}EGVRDLKELPSK^{132} (Fig. 1), the epitope region of the protein (Satoh et al., 2010), was obtained from rabbit blood through subcutaneous injection, administered five times, of the synthetically produced Fag e 2 peptide-KLH (keyhole limpet hemocyanin) conjugate, using the same protocol (Fag e 2 peptide-pAb).

2.4. Preparation of buckwheat extract

For testing purposes, two different extracts were used: a salt-soluble protein extract of Kitawase buckwheat (*Fagopyrum esculentum* Moench cv Kitawase) and an allergen scratch extract called 'Torii' (buckwheat flour) (Torii pharm, Tokyo, Japan). To prepare the salt-soluble extract of Kitawase buckwheat, mature buckwheat seeds were ground twice with a Multi Beads Shocker (Yasui Kikai Corp., Osaka, Japan) at 2,000 rpm for 60 s. Seventy-five milligrams of the powdered sample were mixed with 3 ml of PBS by rotating at 4 °C for one hour with a Rotator (RT-50, TAITEC Corp., Saitama, Japan). The protein solutions extracted with PBS were then centrifuged at 12,000 g for 5 min at 4 °C, and the supernatants were collected as salt-soluble proteins. The protein concentrations were determined with a Pierce™ BCA Protein Assay Reagent Kit (#23225, Thermo Fisher Scientific Inc., Waltham, MA, USA). The protein solutions were stored in aliquots at −80℃ until use.

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CLUSTAL 2.1 multiple sequence alignment


BW10kDa         MKLFLILAAASLLIVASHADS----------QMRSKCRKQMRMMEPQLEQCEGYMTMDM    49
Fag_e_2         MKLFIILATATLLIAATQATYPRDEGFDLGETQMSSKCMRQVKMNEPHLKKCNRYIAMDI    60
                ****:***:*:***.*::*             ** *** :*::* **:*::*: *::**:


BW10kDa         MDD------DSMRGRECRSEESCMRGCCLAMKEMDDECMCEWMKMMVQQQRGEMGEEDMR   103
Fag_e_2         LDDKYAEALSRVEGEGCKSEESCMRGCCVAMKEMDDECVCEWMKMMVENQKGRIGERLIK   120
                :**      . :.*. *:**********:*********:********::*:*.:**. ::


BW10kDa         MVMRKMKQLPNKCGMGHMRCHMGIGTRDYE                                133
Fag_e_2         EGVRDLKELPSKCGLSELECGS-RGNRYFV                                149
                  :*.:*:**.***:..:.*    *.* :
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Fig. 1. Comparison of buckwheat 10 kDa allergen (BW10kDa) sequence with Fag e 2. The deduced amino acid sequences of BW10kDa (133 aa) and Fag e 2 (149 aa) have been aligned by using the ClustalW program.

2.5. Western blotting

First, the concentration of the applied sample (BW extract or recombinant protein) was adjusted with PBS to 1 mg/mL, and an equal volume of 2 × Laemmli Sample Buffer (LSB) (Bio-Rad, Hercules, CA, USA) and 1/10 vol of 1 M dithiothreitol solution (DTT) (Nacalai tesque, Kyoto, Japan) were added (Laemmli,1970). After the prepared sample was boiled at 100 °C for 2 min, SDS-electrophoresis was performed with 20 μL of sample per well and 10 μL of rainbow marker (Cytiva, Uppsala, Sweden) added to a 12 % precast gel (for 15 wells, Bio-Rad, Hercules, CA, USA) at a constant current of 20 mA. After completion of electrophoresis, the precast gel removed from the fixation plate was applied to a transfer buffer (Tris/Glycine Transfer buffer containing 10 % methanol) to equilibrate the buffer. The activated 0.2 μm polyvinylidene difluoride membrane (PVDF, Bio-Rad, Herules, CA, USA) was then subjected to overnight electrical transfer at 3 W constant power. After completion of the transfer, the PVDF membrane was immersed in PBS-T (PBS-0.05 % Tween 20), washed under shaking for 5 min, and then immersed in Blocking One (Nacalai tesque, Kyoto, Japan) for 1 h. The cleaned PVDF membrane was cut into 4-mm-wide strips, as necessary. The washed PVDF membrane was immersed in a primary antibody diluted with 5 % Blocking One-PBS-T and shaken at room temperature for 1 h. As for the primary antibody, pAbs were used at a 4000-fold dilution and mAbs at a double-fold dilution. After incubation with the primary antibody, the membrane was rinsed twice with PBS-T and washed under shaking three times for 10 min each. Then, the membrane was immersed in a secondary antibody diluted with 5 % Blocking One-PBS-T and incubated under shaking at room temperature for 1 h. As the secondary antibody, anti-mouse HRP-labeled secondary antibody (Thermo Fisher Scientific Co. USA) was used in 1000-fold dilution for the mAb experiments and anti-rabbit HRP-labeled secondary antibody (Bio-Rad, Herules, CA, USA) was used in 4000-fold dilution for the pAb experiments. After incubation with the secondary antibody, the membrane was rinsed twice with PBS-T and then washed three times under shaking for 10 min and then incubated with Amersham ECL Prime Western Blotting Detection Reagent (Cytiva, Uppsala, Sweden) for about 1 min to detect chemiluminescence with Amersham Imager 680. All experiments were performed at least twice to confirm reproducibility.

2.6. Spot membrane assay (epitope analysis)

The epitopes of the antibodies were analyzed by reacting a 12-amino acid sequence of BW10kDa with a spot membrane (BW10kDa-PepSpots membrane) in which 63 spots staggered from each other by 2 residues, were arranged.

To prevent hydrophobic peptides from precipitating, the PepSpots membrane (JPT, Berlin, Germany) was rinsed with a small amount of methanol for 5 min and then washed 3 times with TBS-T (0.05 % Tween 20 in TBS (50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, Nacalai tesque, Kyoto, Japan)) for 10 min each. Then, it was blocked with Blocking One (Nacalai tesque, Kyoto, Japan) and allowed to stand under shaking at room temperature for more than 2 h. It was then rinsed twice with TBS-T, and then shaken 3 times for 10 min to reduce the background. The spot membrane was placed in a hybrid bag (Cosmo Bio, Tokyo, Japan) cut according to its size, immersed in a primary antibody diluted with 5 % Blocking One-TBS-T, and incubated overnight under shaking at 4℃. As the primary antibody, the mAbs were used as the stock solution or in 2-fold dilution, and pAbs were used in 4000-fold dilution. After the primary antibody was collected, the spot membrane was rinsed twice with TBS-T and washed three times for 10 min. Then spot membrane was immersed in a secondary antibody diluted with 5 % Blocking One-TBS-T, and incubated under shaking at room temperature for 2 h. As the secondary antibody, anti-mouse HRP-labeled secondary antibody was used in 1,000-fold dilution for the mAb experiments and anti-rabbit HRP-labeled secondary antibody was used in 1000-fold dilution for the pAb experiments. Then, the spot membrane was washed 3 times with TBS-T for 10 min and incubated with Amersham ECL Prime Western Blotting Detection Reagent (Cytiva, Uppsala, Sweden) for about 1 min to detect chemiluminescence with Amersham Imager 680.

2.7. Spot membrane regeneration

The spot membrane used was washed with distilled water 3 times for 10 min, washed 3 times with 5 % DMF (dimethylformaldehyde) (Nacalai tesque, Kyoto, Japan) for 10 min, washed again 3 times with distilled water for 10 min, and then washed 3 times with 8 M Urea (Sigma-Aldrich, Basel, Switzerland) solution containing 1 % SDS and 0.1 % 2-mercaptoethanol (2-ME) for at least 10 min, washed with 50 % EtOH (ethanol) and 10 % AcOH (acetic acid) solution 3 times for at least 10 min. After washing, it was rinsed 3 times with distilled water for 10 min, rinsed 3 times with TBS-T for 10 min. Finally, the spot film was rinsed with a substrate solution containing detection reagent, and the reproduction was confirmed by exposure for at least the same time as the original exposure.

3. Results

3.1. Comparison of the deduced amino acid sequences of BW10kDa and Fag e 2

The deduced amino acid sequences of BW10kDa (133 aa) and Fag e 2 (149 aa) were aligned using the ClustalW programme (https://www.genome.jp/tools-bin/clustalw) (Fig. 1). Both BW10kDa and Fag e 2 possess 10 Cys residues, including a conserved motif of 8 Cys residues characteristic of 2S albumin family proteins.

3.2. Western blot analysis

In Fig. 2, rBW10kDa-pAbs (lane1, 2, 3) and rBW10kDa-mAbs (lane 4; 7D6, lane 5; 16F9) exhibited strong reactivity with purified rBW10kDa, while rFag e 2-pAbs (lane 6) and Fag e 2-peptide-pAbs (lane 7) showed weaker reactivity with rBW10kDa.

In Fig. 3, rBW10kDa-pAbs (lane 1, lane 2) reacted with rFag e 2, whereas rBW10kDa-mAbs (lane 3; 7D6, lane 4; 16F9) did not. Both rFag e 2-pAbs (lane 5) and Fag e 2-peptide-pAbs (lane 6) showed reactivity with rFag e 2.

The reactivities of 5 types of rBW10kDa-mAbs with rBW10kDa and rFag e 2 were further analysed in Fig. 4. Purified rBW10kDa was used as the target protein in lanes 1 to 5, and rFag e 2 was used as the target protein in lanes 6 to 10. 5 kinds of rBW10kDa-mAbs were used as

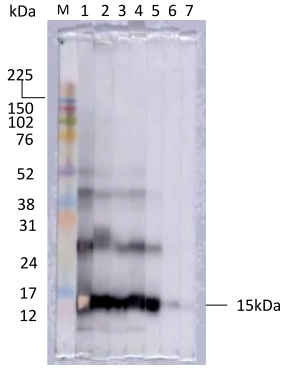

Fig. 2. Western blot analysis of various antibodies to rBW10kDa. Purified rBW10kDa (1 μg/lane) was subjected to 12 % SDS-PAGE and immunoblotted using various animal sera. Lanes 1–3; rBW10kDa-pAb (2000 dil., 4000 dil., 8000 dil. each), Lanes 4–5; rBW10kDa-mAb (7D6, 16F9 each), Lane 6; rFag e 2-pAb (2000 dil.), lane 7; Fag e 2-peptide-pAb (2000 dil.). M; Molecular weight marker. A representative result of two similar experiments is shown.

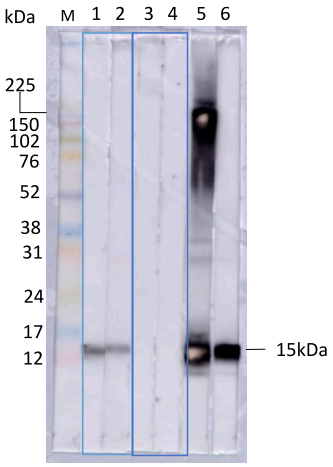

Fig. 3. Western blot analysis of various antibodies to rFag e 2. Purified rFag e 2 (1 μg/lane) was subjected to 12 % SDS-PAGE and immunoblotted using various animal sera. Lanes 1–2; rBW10kDa-pAb (2000 dil., 4000 dil. each), Lanes 3–4; rBW10kDa-mAb (7D6, 16F9, 2 dil. each), Lane 5; rFag e 2- pAb (2000 dil.), lane 6; Fag e 2 −peptide-pAb, (2000 dil.), M; Molecular weight marker. A representative result of two similar experiments is shown.

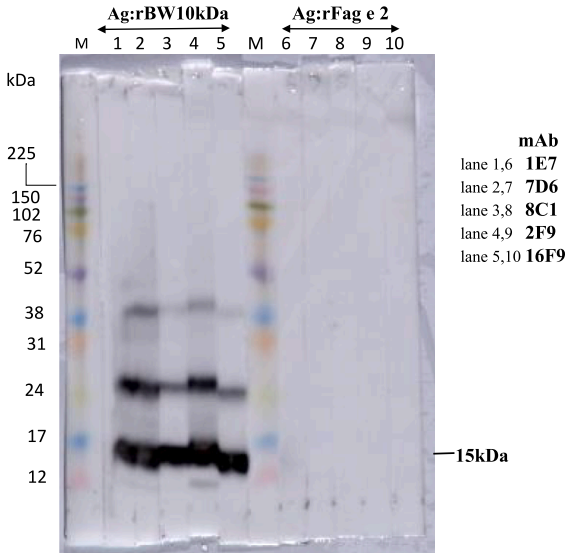

Fig. 4. Western blot analysis of various rBW10kDa-mAb to rBW10kDa and rFag e 2. Purified rBW10kDa (1 μg/lane) (Lanes 1–5) and rFag e 2 (1 μg/lane) (Lanes 6–10) were subjected to 12 % SDS-PAGE and immunoblotted using various rBW10kDa-mAbs. Lanes 1, 6; 1E7 (2 dil.), Lanes 2, 7; 7D6 (2 dil.), Lanes 3, 8; 8C1 (2 dil.), Lanes 4, 9; 2F9 (2 dil.), Lanes 5, 10; 16F9 (2 dil.), M; molecular weight marker. A representative result of two similar experiments is shown.

follows; 1E7 in lanes 1 and 6, 7D6 in lanes 2 and 7, 8C1 in lanes 3 and 8, 2F9 in lanes 4 and 9, and 16F9 in lanes 5 and 10. All rBW10kDa-mAbs reacted with rBW10kDa(lanes 1–5), and bands were seen at 15 kDa position, but not reacted with rFag e 2 (lanes 6–10), clearly distinguishing between the two proteins.

In Fig. 5, the responsiveness of the rBW10kDa-mAbs to two types of crude buckwheat extracts (a commercially available allergen scratch extract and kitawase wild strain extract) were evaluated. Fig. 5 **(a)** depicts the outcomes using allergen scratch extract "Torii" (buckwheat flour) as the target protein and 5 types of rBW10kDa-mAbs as the primary antibody, as follows: 1E7 in lane 1, 7D6 in lane 2, 8C1 in lane 3, 2F9 in lane 4, and 16F9 in lane 5. For lanes 1 to 4, involving type 1 mAbs, reactivity was observed for both the 15-kDa protein and the 10-kDa protein, while in lane 5, with a type 2 mAb, reactivity was

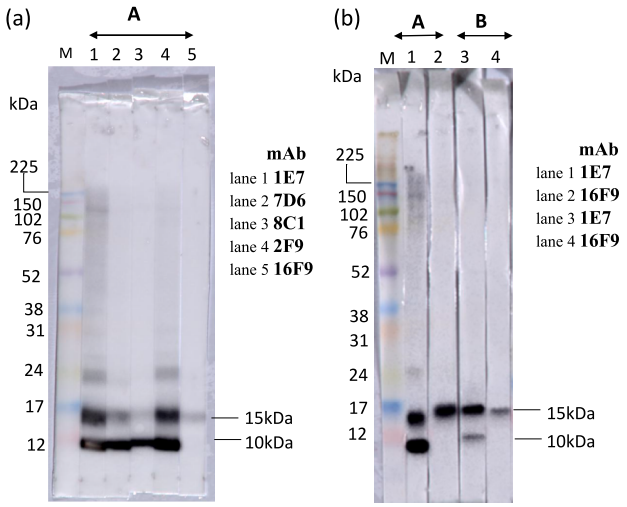

Fig. 5. Western blot analysis of various rBW10kDa-mAbs reacting with allergy-diagnostic or wild-type BW extract. a) Allergy-diagnostic (A) BW extract was subjected to 12 % SDS-PAGE and immunoblotted with 5 kinds of rBW10kDa-mAbs (Lane 1; 1E7, Lane 2; 7D6, Lane 3; 8C1, Lane 4; 2F9, Lane 5; 16F9, 2dil. each). Allergy-diagnostic (A) or wild-type (B) BW extract was subjected to 12 % SDS-PAGE immunoblotted using 2 kinds of rBW10kDa-mAbs (Lanes 1, 3; 1E7, Lanes 2, 4; 16F9, 2 dil. each). M; molecular weight marker. Both a) and b) show representative results of two similar exprements.

observed solely for the 15-kDa protein. Fig. 5**(b)** shows the results obtained using an allergen scratch BW extract in lanes 1 and 2 and wild-type BW extract in lanes 3 and 4. Primary antibodies were 1E7 (type 1) in lanes 1 and 3 and 16F9 (type 2) in lanes 2 and 4. In lanes 1 and 3, reactions were observed for the 15-kDa protein and the 10-kDa protein, whereas in lanes 2 and 4, reactions were detected only for the 15-kDa protein. Notably, when the wild-type BW extract was employed, both the full-length 15-kDa protein and the 10-kDa protein were recognized, akin to the recognition observed with the Torii allergen scratch extract. From these findings, it is suggested that the 10-kDa protein might result from partial cleavage of the full-length 15-kDa protein by a specific protease as a post-translational modification (Katsube-Tanaka and Monshi, 2022; Shewry et al., 1995). Additionally, the ratio of cleaved to full-length proteins appears to fluctuate depending on the type of BW. Notably, the 10-kDa protein was scarcely detectable in the purified recombinant proteins, possibly due to the absence of the post-translational modification observed in plants not occuring in the *E. coli*.

Finally, as shown in **Fig. S1**, demonstrated the reactivity of rBW10kDa-pAbs with the wild-type BW extract, indicating recognition of the 15-kDa protein and the 10-kDa proteins.

3.3. Epitope analysis

In the experiment using the BW10kDa-PepSpots membrane, epitope analysis of the pAbs and mAbs was performed, and the results are shown in Fig. 6 and Table 1.

As antibodies, 5 types of rBW10kDa-mAbs (1E7, 7D6, 8C1, 2F9, and 16F9) and rBW10kDa-pAbs were used. In Fig. 6, the pAbs reacted with multiple spots over the entire length, but the mAbs reacted only with spots close to the C-terminus.

Table 1 shows a summary of the reactions of the antibodies with each spot on the SPOT membrane shown in Fig. 6.

Differences in the spot reactivities between the pAbs and mAbs confirmed differences in the epitopes recognized; the mAbs showed different reactivities between Fag e 2 and BW10kDa and with spots showing low homology between the two proteins (especially spot No. 47–50 (Type 1 mAbs; 1E7, 8C1), spot No. 58–62 (Type 1 mAbs; 7D6, 2F9), spot No. 27–28 (Type 2 mAb; 16F9)).

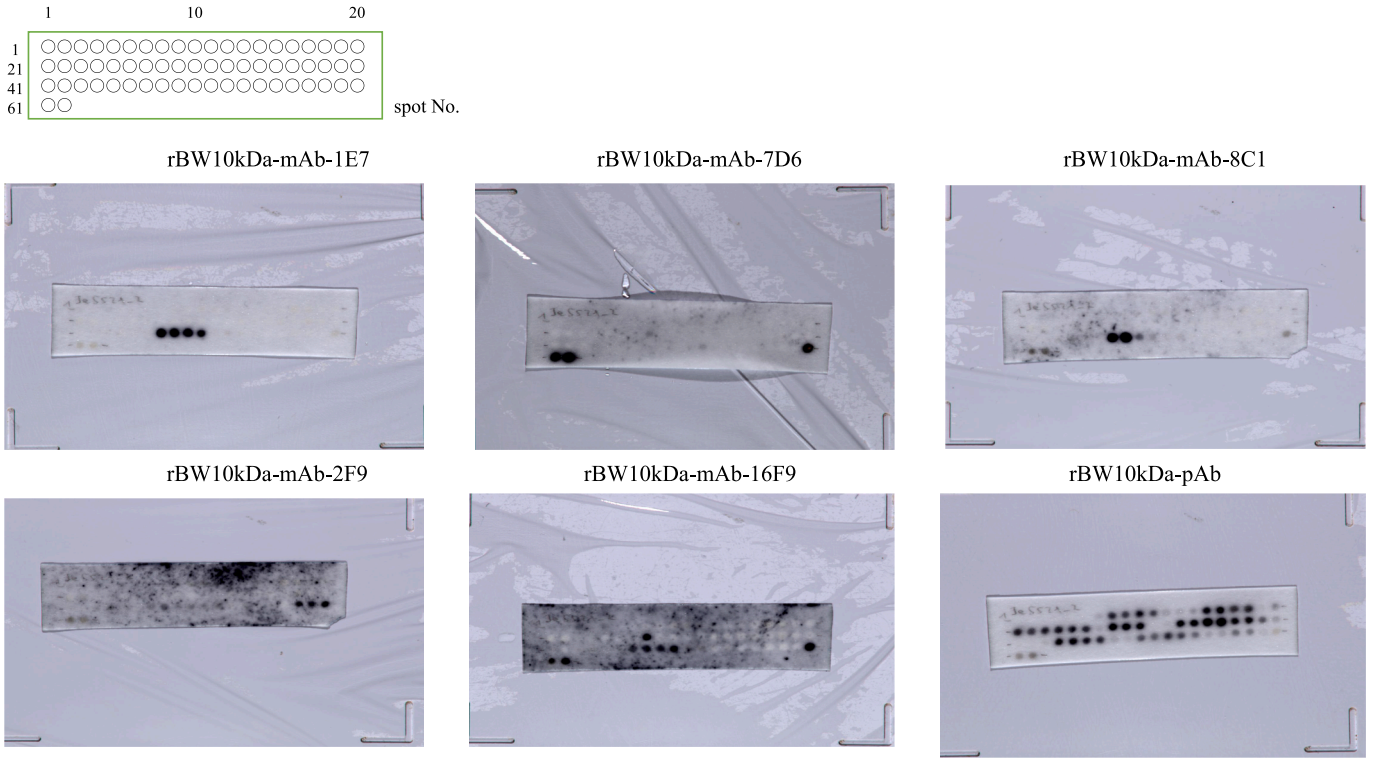

Fig. 6. Results of the SPOT assay using a BW10kDa-Pepspots membrane. The membrane containing spots with overlapping peptides was probed with 5 kinds of rBW10kDa-mAbs (1E7, 7D6, 8C1, 2F9, 16F9, undiluted solution) or rBW10kDa-pAb (4000 dil).

The similarity of the sequence of the BW10kDa-derived peptide (12 amino acids) used for the SPOT analysis and the peptide of the corresponding portion of Fag e 2 (12 amino acids), which was classified into three grade (high homology: 9 or more amino acids are the same; middle homology: 5 to 8 amino acids are the same; low homology: 4 or less amino acids are the same), is shown in the second column from the right in Table 1.

As the type 1 mAbs recognized the epitopes near the C-terminal side region, the 10-kDa post-translated cleaved protein seems to be a large C-terminus subunit. And as the type 2 mAb recognized the 15-kDa full length protein, it would seem that the reaction site of the cleavage protease is just behind a site recognized by the type 2 mAb.

4. Discussion

BW10kDa (Matsumoto et al., 2004), identified as a buckwheat allergen, belongs to the 2S-albumin protein family, akin to Fag e 2 (Koyano et al., 2006), another buckwheat allergen. The results of blast homology search of the deduced amino acid sequences of Fag e 2 (Genbank accession No. DQ304682) and BW10kDa (Genbank accession No. AB055892) are shown in Fig. 1, and it is evident from the figure that the homology between full-length Fag e 2 and BW10kDa was more than 50 %. Especially, the similarity of Fag e 2 to BW10kDa in length was 54 % (72 amino acids/133 amino acids).

In this investigation, we aimed to differentiate between BW10kDa and Fag e 2 using antibodies, and to evaluate BW10kDa expression levels in buckwheat extract.

For the first time, we generated polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs) specifically targeting rBW10kDa. While rBW10kDa-pAbs did not distinguish rBW10kDa from rFag e 2, rBW10kDa-mAbs exclusively reacted with rBW10kDa as depicted in Fig. 2, Fig. 3, and Fig. 4. Consequently, employing rBW10kDa-mAbs facilitated the detection and differentiation of BW10kDa and Fag e 2, both implicated as causative allergens in buckwheat allergy, thereby aiding in confirming of allergen-free buckwheat. Moreover, the similar reactivity of rBW10kDa-mAbs with proteins in both allergy-diagnostic and wild buckwheat extracts confirmed the presence of BW10kDa protein in the allergy-diagnostic buckwheat extract (Fig. 5).

Our findings suggest the potential identification of patients responsive to BW10kDa protein. Recent reports by Yasui et al. (2016) and Katsube-Tanaka and Monshi (2022) highlighted the presence of 2S-albumin family genes in common buckwheat, iudicating that BW10kDa protein is encoded by g11 and g28 genes, with an expression level of approximately 20 μg/seed; while Fag e 2 is encoded by g14 and present at about 60 μg/seed.

Notably, IgE antibodies binding to BW10kDa are detected at a high frequency (57 %) in the serum of patients with buckwheat allergy (Matsumoto et al., 2004). The particular region of the C-terminal site of BW10kDa recognized by rBW10kDa-mAbs may correspond to the epitope recognized by the IgE antibodies in patients with allergy to BW10kDa.

This study successfully produced mAbs capable of discerning between Fag e 2 and BW10kDa, facilitating the confirmation of allergen-free buckwheat, diagnosis of buckwheat allergy and assessment of the involvement of BW10kDa in anaphylactic reactions caused by buckwheat consumption. These findings lay the groundwork for future development of buckwheat varieties.

5. Conclusion

The detailed analyses of BW10kDa have been performed by mAbs to rBW10kDa. The mAbs recognized the unique structure of BW10kDa, and these were possible to distinguish between Fag e 2 and BW10 kDa. Thus by using mAbs, it was possible to consider the involvement of BW10kDa alone in anaphylactic reaction, analyze the expression level of BW10kDa in buckwheat extracts and confirmation of allergen-free buckwheat.

CRediT authorship contribution statement

Masaya Kimura: Investigation. **Rie Satoh:** Investigation. **Reiko**

Table 1
BW10kDa peptide array experiment.

spot No	sequence	Start aa no	End aa no	poly Ab pos	mAb 1E7 pos	mAb 7D6 pos	mAb 8C1 pos	mAb 2F9 pos	mAb 16F9 pos	Similarity to Fag e 2	ref
1	MKLFLILAAASL	1	12								signal peptide
2	LFLILAAASLLI	3	14								signal peptide
3	LILAAASLLIVA	5	16								signal peptide
4	LAAASLLIVASH	7	18								signal peptide
5	AASLLIVASHAD	9	20								signal peptide
6	SLLIVASHADSQ	11	22								
7	LIVASHADSQMR	13	24								
8	VASHADSQMRSK	15	26	○						middle	
9	SHADSQMRSKCR	17	28	○						middle	
10	ADSQMRSKCRKQ	19	30	○						middle	
11	SQMRSKCRKQMR	21	32	○						middle	
12	MRSKCRKQMRMM	23	34								
13	SKCRKQMRMMEP	25	36								
14	CRKQMRMMEPQL	27	38								
15	KQMRMMEPQLEQ	29	40	○						middle	
16	MRMMEPQLEQCE	31	42	○						middle	
17	MMEPQLEQCEGY	33	44	○						middle	
18	EPQLEQCEGYMT	35	46	○						middle	
19	QLEQCEGYMTMD	37	48								
20	EQCEGYMTMDMM	39	50								
21	CEGYMTMDMMDD	41	52	○						middle	
22	GYMTMDMMDDDS	43	54	○						middle	
23	MTMDMMDDDSMR	45	56	○						low	
24	MDMMDDDSMRGR	47	58	○						middle	
25	MMDDDSMRGREC	49	60	○						low	
26	DDDSMRGRECRS	51	62	○							
27	DSMRGRECRSEE	53	64						○	middle	
28	MRGRECRSEESC	55	66	○					○	middle	
29	GRECRSEESCMR	57	68	○						middle	
30	ECRSEESCMRGC	59	70	○						high	
31	RSEESCMRGCCL	61	72								
32	EESCMRGCCLAM	63	74								
33	SCMRGCCLAMKE	65	76	○						high	
34	MRGCCLAMKEMD	67	78	○						high	
35	GCCLAMKEMDDE	69	80	○						high	
36	CLAMKEMDDECM	71	82	○						high	
37	AMKEMDDECMCE	73	84	○						high	
38	KEMDDECMCEWM	75	86	○						high	
39	MDDECMCEWMKM	77	88								
40	DECMCEWMKMMV	79	90								
41	CMCEWMKMMVQQ	81	92								
42	CEWMKMMVQQQR	83	94								
43	WMKMMVQQQRGE	85	96								
44	KMMVQQQRGEMG	87	98	○						middle	
45	MVQQQRGEMGEE	89	100	○						middle	
46	QQQRGEMGEEDM	91	102	○						low	
47	QRGEMGEEDMRM	93	104	○	○		○			low	
48	GEMGEEDMRMVM	95	106		○		○			low	
49	MGEEDMRMVMRK	97	108		○		○			low	
50	EEDMRMVMRKMK	99	110	○	○					low	
51	DMRMVMRKMKQL	101	112	○						low	
52	RMVMRKMKQLPN	103	114	○						low	
53	VMRKMKQLPNKC	105	116	○						middle	
54	RKMKQLPNKCGM	107	118	○						middle	
55	MKQLPNKCGMGH	109	120								
56	QLPNKCGMGHMR	111	122								
57	PNKCGMGHMRCH	113	124								
58	KCGMGHMRCHMG	115	126					○			
59	GMGHMRCHMGIG	117	128					○			
60	GHMRCHMGIGTR	119	130			○		○		low	
61	MRCHMGIGTRDY	121	132			○		○		low	
62	RCHMGIGTRDYE	122	133			○		○		low	

Similarity to Fag e 2 (number of matched amino acids/12 amino acids): high;>=9/12, middle; <=8/12 and >=5/12, low;<=4/12.

Teshima: Writing – original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary material

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