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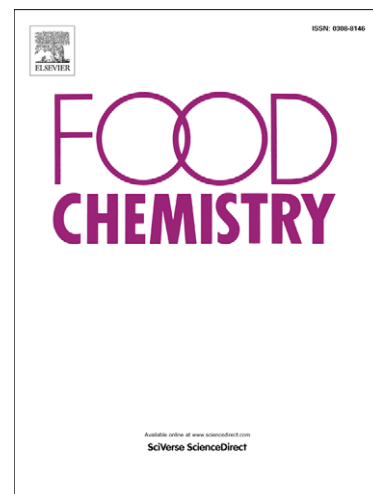
Tandem repeat inserts in 13S globulin subunits, the major allergenic storage protein of common buckwheat (*Fagopyrum esculentum* Moench) seeds

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PII: S0308-8146(11)01829-2
DOI: [10.1016/j.foodchem.2011.12.056](https://doi.org/10.1016/j.foodchem.2011.12.056)
Reference: FOCH 11925

To appear in: *Food Chemistry*

Received Date: 29 July 2011
Revised Date: 21 October 2011
Accepted Date: 14 December 2011



Please cite this article as: Khan, N., Takahashi, Y., Katsube-Tanaka, T., Tandem repeat inserts in 13S globulin subunits, the major allergenic storage protein of common buckwheat (*Fagopyrum esculentum* Moench) seeds, *Food Chemistry* (2011), doi: [10.1016/j.foodchem.2011.12.056](https://doi.org/10.1016/j.foodchem.2011.12.056)

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1 **Title**

2 Tandem repeat inserts in 13S globulin subunits, the major allergenic storage protein of
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ABSTRACT

The prevalent allergen of common buckwheat (*Fagopyrum esculentum* Moench) is β polypeptide of the most abundant storage protein, 13S globulin. Because the 13S globulin is composed of multiple diversified subunits, α polypeptide, the counterpart of β polypeptide in 13S globulin subunits, was characterized in depth to better understand this allergenic protein. Most α polypeptides were reactive to antibodies for 13S globulin-related rice glutelin and soybean glycinin and seemed to be methionine-poor subunits. Four new methionine-poor subunits with 0, 2, 4 and 6 tandem repeats were identified. The α polypeptides showed a comparatively significant variation in size, which was largely attributed to the different lengths of the tandem repeat inserts in methionine-poor subunits. Because the tandem repeat region was hydrophilic with many arginyl residues, digestibilities against trypsin, that is one of the critical characteristics for allergens, were different between the subunits with and without the tandem repeat.

Keywords

Common buckwheat; Seed storage protein; Subunit composition; 13S globulin; Allergen.

Abbreviations

IPG, immobilized pH gradient; Met, methionine; Cys, cysteine.

1. Introduction

Common buckwheat (*Fagopyrum esculentum* Moench) seed proteins are recognized as valuable proteins with high nutritional and biological values (Pomeranz & Robbins, 1972; Bonafaccia, Marocchini & Kreft, 2003). Buckwheat seed proteins are considered health-promoting proteins with many physiological functions, such as the ability to lower blood cholesterol (Kayashita, Shimaoka & Nakajoh, 1995) and to prevent accumulation of fat, constipation (Kayashita, Shimaoka, Nakajoh & Kato, 1996), mammary carcinogenesis and colon carcinogenesis (Liu et al., 2001), and to suppress gallstone formation and plasma cholesterol (Tomotake, Shimaoka, Kayashita, Yokoyama, Nakajoh & Kato, 2000). However, despite the health-promoting benefits of the buckwheat proteins, buckwheat seed proteins are also allergenic to human beings (Wieslander & Norbaeck, 2001). The allergy to buckwheat protein is now becoming a great concern to public health due to the increased use of buckwheat as a health food (Wijngaard & Arendt, 2006). The elimination or mitigation of its allergenic proteins, as is needed with the allergenic proteins of rice and soybean (Herman, Helm, Jung & Kinney, 2003; Tada et al., 1996), is highly desired for the enhancement and potential utilization of buckwheat as a food crop.

The allergens in buckwheat have been identified and characterized molecularly by several research groups. Even though the allergens varied among patient sera, Park et al. (2000) revealed that the 9-, 16-, 19- and 24-kDa proteins were the most prevalent allergens and that the 30-, 43- and 67-kDa proteins were the less prevalent allergens. Particularly, the 9-kDa protein (identical to 6-kDa allergen by Park, Abe, Kimura, Urisu & Yamasaki (1997)) is a trypsin inhibitor called BWI-2a/2b. The 16-kDa protein shares

38% homology with the α -amylase/trypsin inhibitor of finger millet (Park et al., 2000). The 16- to 18-kDa protein is referred to as a Fag e 2 and shows similarities to the Ara h 2 (peanut) and Ric c 1/Ric c 3 (castor bean) allergens (Yoshioka, Yasueda & Adachi, 2004). The 19-kDa protein has a 50% homology with rice's 19-kDa α -globulin (a member of the 2S albumin) (Park et al., 2000). The 24-kDa protein (named Fag e 1), which is recognized as one of the most significant allergens by researchers, is the β polypeptide of the 13S globulin (Nagata et al., 2000). Although the exploration for Fag e 1-null genotype has been unsuccessful (Nair & Adachi, 1999; Maruyama-Funatsuki, Fujino, Suzuki & Funatsuki, 2004), the finding of varietal variation in the content of Fag e 1 (Maruyama-Funatsuki et al., 2004) has encouraged us to minutely characterize the 13S globulin.

Buckwheat seeds are 8.5% to 18.9% protein (Krkošková & Mrázová, 2005). The 13S globulin is the most abundant protein accounting for approximately 43% of the total seed protein (Radovic', Maksimovic' & Varkonji-Gas'ic', 1996). The 13S globulin is salt-soluble and resembles the legumin-like seed storage protein of other species, such as rice glutelin and soybean glycinin (members of the 11S globulin family) in terms of its sedimentation constant, amino acid homology and similarities in biosynthetic and accumulation processes (Radovic' et al., 1996; Derbyshire, Wright & Boulter, 1976; Dickinson, Hussein & Nielsen, 1989). Like other legumin-like seed storage proteins, the buckwheat 13S globulin is composed of multiple subunits, each of which contains acidic (α) and basic (β) polypeptides covalently linked by a disulfide bond.

Because the multiple subunits have different characteristics from each other, the subunit composition of the seed storage protein affects the nutritional quality of rice

glutelin (Kawakatsu, Hirose, Yasuda & Takaiwa, 2010), other storage proteins (Fujiwara & Otsu, 2005), and the physicochemical quality of the soybean food products with altered glycinin subunit composition (Poysa, Woodrow & Yu, 2006). Although the subunit composition of seed storage proteins is varied by plant nutrition (i.e. by the availability of nitrogen and sulfur) (Tabe, Hagan & Higgins, 2002), even genotypical variation in the subunit composition of buckwheat 13S globulin has seldom been of concern, except in some studies by Cepkova and Dvoracek (2006), Rogl and Javornik (1996), and Bonafaccia, Acquistucci and Luthar (1994). To date, only four types of 13S globulin subunits have been identified.

In our current paper, we identified and characterized the α polypeptides of the buckwheat 13S globulin by SDS-PAGE, 2D-PAGE, western blotting with antibodies against the 13S globulin-related rice glutelin and soybean glycinin, and PCR of genomic DNA. Various lengths of tandem repeats inserted in four newly found subunits, in conjunction with those in the three known subunits, were suggested to be mostly responsible for the large diversity of the α polypeptides. And then, the relationship between the structure and the stability to proteinase was analyzed. This is the first report, which comprehensively argued the diversity in the primary structure and the distinct digestibility of the buckwheat 13S globulin subunits.

2. Materials and methods

2.1. Plant materials and preparation of the 13S globulin

Flour and seeds of common buckwheat cultivar ‘Shinano-ichigo’ were obtained from a local milling company in the Nagano prefecture, Japan and a local nursery company in the Shiga prefecture, Japan, respectively. Note that the commercial flour was made of uncounted number of ground seeds and was expected to show averaged and practical characteristics in terms of protein composition. Tartary buckwheat seeds of cultivar ‘FT Rotundatiem’ were a gift from the National Agricultural Research Center for Kyushu Okinawa Region. The protein fraction of the 13S globulin was extracted from either commercial flour or a single seed with the extraction buffer (0.035 M potassium phosphate, pH 7.6 and 0.4 M sodium chloride). The 13S globulin was either dissolved in the sample buffer containing 50 mM Tris, pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (w/v) glycerol, and 5% (v/v) 2-mercaptoethanol for SDS-PAGE analysis or dissolved in the lysis buffer containing 9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, and 5% Bio-Lyte 3-10 (Bio-Rad, USA) for 2D-PAGE analysis.

2.2. SDS-PAGE, 2D-PAGE and Western blot analyses

SDS-PAGE was performed according to the procedure by Laemmli (1970) with minor modifications, using 14% T acrylamide gel at a constant voltage of 200 V. IEF was performed with IPG strips of pH ranges 3-10 or 4-7 (BioRad, USA) with a 7.5-cm length according to the manufacturer’s instruction. Electrophoresis in the first dimension (IEF) was performed at 200 V for 10 minutes, 400 V for 10 minutes, 1000 V for 10 minutes, and 1500 V for 12 hours. Immunoblotting was performed according to the method

mentioned in our previous report (Khan, Katsube-Tanaka, Iida, Yamaguchi, Nakano & Tsujimoto, 2008). Briefly, the proteins resolved by SDS-PAGE and 2D-PAGE were electrophoretically transferred to nitrocellulose membrane. The membrane was incubated in blocking solution (0.15 M NaCl, 5% (w/v) skimmed milk, 0.05% (v/v) Tween-20 and 0.04 M Sodium-phosphate buffer pH 7.4) with gentle shaking for 1 hour at room temperature. Then the membrane was incubated with primary antibody of anti-soybean glycinin and anti-rice glutelin individually or step-by-step (Khan et al., 2008) at room temperature for 1 hour. Following this the membrane was thoroughly washed three times for 20 minutes with TBST (0.02 M Tris pH 7.5, 0.9% (w/v) NaCl, 0.05% (v/v) Tween-20) and then incubated with secondary goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, USA) for 1 hour at room temperature. Subsequently, the membrane was washed three times for 20 minutes with TBST. Finally, color development was accomplished with NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate). The detection was stopped in deionized water and the membrane was air dried prior to scanning. The antigens of anti-soybean glycinin and anti-rice glutelin antibodies were expressed in and purified from *Escherichia coli* cells as recombinant proteins and used for immunization of rabbit (Katsube et al., 1999; Katsube-Tanaka et al., 2004).

2.3. Genomic DNA extraction and PCR Analysis

Genomic DNA extraction from the 'Shinano-ichigo' single seeds and PCR analysis were performed according to a kit (Ampdirect plus, Shimadzu, Japan). The primers used over the tandem repeat region around 433-663 bp from the 5'-end of the

GenBank accession gene D87980 were as follows: forward (left1) was AGGATG(C/T)CCGGAGAC(A/G)T(A/T)CCA and reverse (right1) was CTAACGTTC(C/T)CATCGAGCTG for the methionine-poor (Met-poor) subunits. The PCR reaction was performed in thermal cycler (Takara Standard PCR, Japan). The DNA template was initially denatured for 1 minute at 96 °C followed by 44 reaction cycles each consisting of denaturation for 30 seconds at 94 °C, annealing for 1 minute at 60 °C, elongation for 1 minute at 72 °C and finally the extension reaction was done for 7 minutes at 72°C. The PCR products were electrophoresed on 1% agarose gel containing ethidium bromide and the gel was visualized and photographed under UV transilluminator. Then the PCR amplified bands were purified from agarose gel using DNA purification kit (Easy Trap, Takara, Japan), cloned and sequenced. Samples for sequencing reactions were prepared according to DTCS Quick Start kit (Beckman, USA). Sequencing was done with a Beckman sequencer according to the instruction manual of manufacturer.

2.4. Trypsin digestion

Globulin fractions extracted from the 'Shinano-ichigo' buckwheat flour were incubated with 1/100 (w/w) sequencing grade trypsin (Promega, USA) in 50 mM ammonium bicarbonate (pH 7.8) for two hours at 37 °C according to the manufacturer's instruction. Aliquots taken at a given times (5, 15, 30, 60 and 120 minutes) were boiled for 5 minutes to terminate the digestion reaction, electrophoresed, and compared by CBB staining and western blotting with the anti-glutelin antibody.

2.5. TOF/MS analysis

CBB stained single bands of the 13S globulin α polypeptide, α_3 , were cut out from SDS-PAGE gel (10% T) and washed three times for 30 minutes with distilled water. Then the bands were transferred to dialysis tube (Wako chemicals, Japan) and electroeluted using elution buffer containing 0.1% SDS, 16.7 mM Tris and 127.9 mM glycine at 135 V for about 60 minutes. The eluted proteins were desalted and concentrated by a spin column with ultrafiltration membrane (Amicon Ultra 0.5ml 10k, Millipore, USA). The protein sample was precipitated by trichloroacetic acid and acetone and dissolved in 1% (v/v) formic acid. After final yield and purity check by SDS-PAGE, the sample was subjected with saturated sinapinic acid solution to MALDI- TOF/MS (autoflex III, Bruker, Germany) with bovine serum albumin (66.4 kDa) as a calibration standard.

3. Results

3.1. Comparison of 13S globulin α polypeptides in the commercial flour of common buckwheat and tartary buckwheat seeds

The 13S globulin fractions from commercial flour of common buckwheat and tartary buckwheat seeds, which are autogamous and homogeneous were resolved by SDS-PAGE, and subsequent subunit discrimination was accomplished by immunodetection using anti-glycinin and anti-glutelin antibodies (Fig. 1). Although banding patterns were different among detection methods, the α polypeptides of common buckwheat cv. 'Shinano-ichigo' were separated into at least 9 major bands (a2-a10) and those of tartary buckwheat were into 7 major bands (ax, a1, a6-a10). A large molecular size variation was observed in both common buckwheat and tartary buckwheat and the α polypeptides of common buckwheat were ranged from 30 to 47 kDa according to the mobility in SDS-PAGE. The bands ax, a1-a4 looked like a single band, while the other lower bands seemed to consist of multiple polypeptides. The western blot data demonstrated the anti-glutelin reacted with most α polypeptides as well as or even stronger than the anti-glycinin did. However, the a7 band reacted relatively strongly with anti-glycinin, whereas the a8 of common buckwheat and the a10 of tartary buckwheat bands didn't react with anti-glycinin. The a9 band of common buckwheat scarcely reacted with both antibodies even though the a9 band of common buckwheat was clearly detected by CBB. The reactivity of anti-glycinin was also low to β polypeptides.

3.2. Characterization of 13S globulin α polypeptides in Shinano-ichigo commercial flour

For a detailed understanding in the variations of the 13S globulin α polypeptides, 13S globulin α polypeptides from 'Shinano-ichigo' commercial flour were analyzed by two dimensional polyacrylamide gel electrophoresis using anti-glycinin and anti-glutelin antibodies (Fig. 2). Immobilized pH gradient (IPG) strips were used for the separation of 13S globulin α polypeptides in the first dimension electrophoresis. Because relatively less divergence in pI s of the 13S globulin α polypeptides, spot congestion resulted when broad range IPG strips (pH 3-10) were used (Fig. 2A). The use of narrow range IPG strips (pH 4-7) enabled improved separation of the 13S globulin α polypeptides, even though the 13S globulin β polypeptides were run off the IPG strip. The major 8 bands recorded on SDS-PAGE gels (Fig. 1) were resolved into several distinct spots with different pI values and with slightly different molecular weights on 2D-PAGE gels, forming a horizontal streak. The horizontal streak of spots might be due to post-translational modification of the polypeptides, and such results were modifications reported with the rice glutelin and soybean glycinin polypeptides (Khan et al., 2008; Natarajan, Xu, Bae, Caperna & Garrett, 2006). Therefore, each horizontal streak of spots was collectively named, instead of naming each spot individually in this study (Fig. 2). The band a6 dissociated into not only similar pI spots to those of other bands but also higher pI spots. The spots corresponding to the band a10 could not be detected by silver staining. When the spots were examined with the anti-glycinin and anti-glutelin antibodies, most spots reacted against either of two antibodies (Figs. 2C and 2D). However, the spot a9 was almost non-reactive with both antibodies.

3.3. PCR for the tandem repeat regions of 13S globulin

A database search and the organization of all retrieved data showed the existence of three types of methionine scarce subunits (Met-poor subunits) and one methionine abounding subunit (Met-rich subunit) (Fig. 3). The three Met-poor subunits contain variable tandem repeat sequences. For example, the GenBank accessions D87982, AF152003, and D87980 have one, three and five tandem repeats respectively, whereas the Met-rich subunit of GenBank accession AY256960 has no tandem repeat sequence (Fig. 3). Because the number of types observed in the SDS-PAGE of the α polypeptides were greater than the number of known subunits, PCR primers were designed at conserved positions (horizontal arrows, Fig. 3) over the region containing the tandem repeats of the Met-poor subunit to explore novel genes. The PCR amplification with the crude genomic DNA extracted from 8 single seeds collectively produced seven types of bands, which were named mp2-mp8 (Fig. 4A). Note that the amplified fragment pattern was quite different seed by seed because of cross-fertilization nature of common buckwheat even within a single variety. The DNA sequences of the representative amplified bands (mp2-mp8) were determined and aligned with the Met-poor subunits of the GenBank accessions: D87980, AF152003 and D87982. The sequence analysis revealed that the clones for bands mp3, mp5 and mp7 correspond to D87980, AF152003 and D87982, respectively. Additionally, the clones for bands mp2, mp4, mp6 and mp8 were from novel genes with 6, 4, 2 and 0 tandem repeats, respectively (Fig. 4A, the numbers in the parentheses following the amplified bands indicate the number of tandem repeats).

3.4. Correlation of band patterns between PCR and SDS-PAGE

The intensities and occurrence frequencies of the PCR amplified bands were varied among the band types. The bands for mp2 and mp6 were scarcely detected, probably due to the low gene doses, frequencies, and primer mismatches (Fig. 4A). However, much higher intensities and occurrence frequencies were observed for the amplified bands of mp3, mp5, mp7 and mp8. When the PCR band patterns were compared with SDS-PAGE patterns examined using the same seeds with those for PCR analysis, the occurrence pattern of the band mp3 showed good correlation with that of the SDS-PAGE band a3 (Figs. 4A and 4B; marked by arrows). In order to confirm the relationship between the mp3 and the a3 bands, other randomly selected 29 seeds were examined. The mp3 and the a3 bands were absent in seeds No. 11, 13, 14, 16, 17, 19, 21, 23 and 26, while they were present in the other seeds. In addition, the mp2-mp8 bands (Fig. 4A) and a2-a8 bands (Fig. 1) were observed at equally spaced intervals. The above results led us to hypothesize that the a2-a8 bands are correlated with mp2-mp8, respectively. In other words, the bands a2, a3, a4, a5, a6, a7 and a8 have 6, 5, 4, 3, 2, 1, and no tandem repeats, respectively.

The α polypeptides of D87980, AF152003, and D87982 have 5, 3, and 1 tandem repeats and the molecular weights of which are calculated to be 41.3 kDa, 37.5 kDa, and 33.5 kDa, respectively. The molecular weights of the bands a3, a5 and a7 estimated from the SDS-PAGE patterns were 43.6 kDa, 36.6 kDa and 33.4 kDa, respectively and matched well with the above-mentioned calculated size except that of the band a3. In order to confirm the exact molecular weight of the band a3, MALDI-TOF/MS analysis was conducted for electro-eluted intact proteins from the band. The results demonstrated the measured molecular weight of the band was 41.2-41.3 kDa (data not shown).

Therefore, the overestimation for the band a3 size by SDS-PAGE was probably due to the retarded mobility of the polypeptide under a reducing condition.

3.5. Variations of 13S globulin α polypeptides in Shinano-ichigo single seeds

Common buckwheat is an allogamous plant, and previous research has demonstrated that the content of the 13S globulin β polypeptides varied greatly among plant individuals of the same variety (Maruyama-Funatsuki et al., 2004). Therefore, the significant diversity of the 13S globulin α polypeptides was also expected at a single seed level in the analysis of SDS-PAGE and western blotting, using the anti-glycinin and anti-glutelin antibodies. The CBB data revealed greater variations in the electrophoretic banding patterns of the 13S globulin α polypeptides among the individual seeds (Fig. 4D). Among the bands described in Figure 1, the bands for a2, a3 and a4 were clearly detected with fixed molecular sizes and slightly different intensities within each band. Among the 29 single seeds examined, a2, a3 and a4 were detected in 14, 20 and 5 of the seeds, respectively, probably reflecting the gene frequency for each band in this cultivar. In contrast, a5 and smaller sized bands showed greater variations in size and intensity, suggesting expression, accumulation and/or modification of such polypeptides were controlled by complicated heredity. The relationship between the bands detected by CBB and the antibodies was evident, especially for the high molecular weight α polypeptides (i.e., a2-a4), but was not clear for the lower bands of the 13S globulin α polypeptides.

3.6. Amino acid sequences of tandem repeats and trypsin digestion

The amino acid sequences deduced from PCR amplified bands mp2-mp8 were compared with those of D87980, AF152003 and D87982 (Fig. 5). The alignment clearly showed the tandem repeat regions had less diversity, except when it came to the lengths of the bands. However, it is notable that the tandem repeat regions and their vicinities had many arginyl residues, and their hydrophobicities were calculated by a method used by Kyte and Doolittle (1982). The regions had negative score values, indicating the regions are likely to be exposed at the molecular surface. This feature of the tandem repeat regions strongly suggests the regions are susceptible to trypsin.

Trypsin digestions demonstrated that most of the 13S globulin α polypeptides degraded within two hours except for the a8 band, which is likely to have no tandem repeat (Fig. 6). The intensity of the a8 band detected by anti-glutelin stayed nearly constant during the reaction, and no new α polypeptides were detected, suggesting the degradation occurred not only at a tandem repeat region but also with the entire α polypeptide sequence. Notably, the a9 band was rather resistant to digestion according to the CBB staining. As described later, the a9 band seems to be derived from a Met-rich subunit, which has no tandem repeats, has relatively higher cysteine (Cys) contents (Fig. 3), and probably has a higher chance of forming a disulfide bond to make a rigid protein structure. Coincidentally, a portion of the β polypeptides was also partially susceptible to the trypsin digestion.

4. Discussion

4.1. Subunits of storage proteins in buckwheat, rice and soybean

The major seed storage protein of buckwheat is a legumin-like 13S globulin that belongs to the 11S globulin family and contains multiple subunits, such as rice glutelin and soybean glycinin. As is common the case with the 11S globulins, the subunits are synthesized as a precursor form which is then processed into α (acidic) and β (basic) polypeptides in accumulation sites. Although it is not common with glycinin and glutelin, some of the 13S globulin α and β polypeptides of common buckwheat are considered major allergens (Nagata et al., 2000; Nair & Adachi, 1999; Sordet, Culerrier, Granier, Didier & Rougé, 2009; Yoshioka, Ohmoto, Urisu, Mine & Adachi, 2004), which can induce an IgE-mediated hypersensitivity reaction. Unfortunately, little information is available about the structural features related to allergenicity and the subunit composition of the 13S globulin. Therefore, this study was performed to obtain comprehensive information on the structural variations and composition of the 13S globulin subunits by focusing on their α polypeptides, which are the counterpart of the β polypeptide and easier to analyze.

The 13S globulin α polypeptides showed relatively large molecular size variations compared to other seed storage proteins. However, better resolution in IEF was required to precisely separate and identify the α polypeptides. In contrast to soybean glycinin and rice glutelin analyses (Khan et al., 2008; Natarajan et al., 2006; Natarajan et al., 2007; Xu et al., 2007), reports on the 2D-profile of the 13S globulin are very limited, except reports by Radovic' et al. (1996) and Renzetti, Behr, Vogel and Arendt (2008). This is the first report highlighting an improved separation of the spots by using the narrow range IPG

strips (pH 4-7), which was a prerequisite for the diversity assessment of the polypeptides (Fig. 2).

4.2. Distinct reactivities of the two antibodies

Our strategy to use antibodies against the 13S globulin-related proteins, soybean glycinin and rice glutelin, instead of the exact anti-13S globulin, was unique and seemed partly successful because we could categorize the 13S globulin α polypeptides into three types (both antibodies reactive, more anti-glycinin reactive, and non-reactive polypeptides). The evolutionary distances calculated from the amino acid sequences have revealed the lower homology of the 13S globulin subunits against the A1aB1b subunit of the soybean glycinin than against the GluB1 subunit of the rice glutelin (the latter two subunits are an antigen subunit for the antibodies used in this study (Katsube et al., 1999; Katsube-Tanaka et al., 2004)). Meanwhile, the Met-rich subunit was found to be more distant to the glycinin and the glutelin subunit than the Met-poor subunits (data not shown). Further information, such as complete amino acid sequences of all the 13S globulin subunits would give an account of such differences in immunoreactivity.

The calculated molecular weight of the α polypeptide of the Met-rich subunit (28.5 kDa for AY256960) is the lowest among the known 13S globulin subunits. The large variation in the molecular size of the α polypeptides, which was observed as a ladder in the SDS-PAGE, seemed to be explained by various length of the tandem repeats inserted into Met-poor subunits. In addition, the spot a9 has obviously different reactivity against anti-glycinin and anti-glutelin antibodies from the other spots. The above observations led us to assume that the non-reactive a9 spot would correspond to a Met-

rich subunit instead. However, in addition to our assumption, the existence of new unique subunits cannot be ruled out. Therefore, further investigation is needed to identify and characterize this unique spot. It is well known that the sulfur-rich to the sulfur-poor subunit ratio of seed storage proteins is altered by nitrogen and sulfur metabolisms (Fujiwara & Otsu, 2005). If the Met-rich subunit is only the $\alpha 9$ spot that is minor in common buckwheat (Fig. 2), environmental factors affecting the nitrogen and sulfur metabolisms would have less impact on the subunit composition fluctuation of 13S globulin.

4.3. Trypsin resistant Met-poor subunit

In this study, we demonstrated that the α polypeptides of the Met-poor subunits with the insertion of the tandem repeats had increased susceptibilities against trypsin. It has been described that resistance to digestion and processing is one of the more important characteristics for food allergenic proteins (Bannon, 2004). In fact, a common in vitro digestion protocol has been established by internationally collaborative research groups for evaluation of the potential allergenicity (Thomas et al., 2004). Sen, Kopper, Pons, Abraham, Burks and Bannon (2002) showed peanut allergenic protein stability upon proteinase digestion affected IgE-binding epitopes intactness. Yano, Kusada, Kuroda and Kato-Emori (2006) demonstrated a correlation between the disulfide bonding and the protease stability of buckwheat proteins and discussed their allergenicity. Although the tandem repeat region of 13S globulin seemed to be located at the molecular surface, the insertion of the tandem repeats might have induced not only the local but also the overall conformational change of the α polypeptides because no partially degraded α

polypeptides could be observed (Fig. 6). Even though the difference in the digestibility of the β polypeptides was empirically not revealed, the tandem repeat insertion into the α polypeptides might have influenced the β polypeptide conformation as well by a conserved inter-polypeptide disulfide bond with one of the involved Cys residues located 18-residues upstream from the tandem repeats. If our hypothesis is correct, the Met-poor subunits of the 13S globulin may have different digestibilities and distinct potential allergenicities. Reducing the content of the trypsin-resistant subunit, the a8 α polypeptide with no tandem repeats, might be useful for the development of novel buckwheat with lowered allergenicity. Further investigation on IgE binding of buckwheat hypersensitive patients should be required.

4.4. Polymorphism at the single seed level

Most previous studies on the identification and quantification of buckwheat allergens have used buckwheat flours or bulked seeds. However, the analysis at a single seed level is also important for out-crossing common buckwheat because of two major advantages. First, the distinctly large polymorphism in the 13S globulin α polypeptides is easily assessed. Second, rapid and precise identification of novel germplasms containing the desired subunit composition is possible. In summary, the findings described here could contribute to the deep understanding on the structure, function, and evolutionary divergence of the buckwheat allergenic proteins by synergistically using systematic hybridization of recently-developed self-pollinating lines (Woo, Adachi, Jong & Campbell, 1999) and/or screening many other local landraces.

Acknowledgements

This work was supported in part by grants to T.K.-T. from the Ministry of Education, Culture, Sports, Science, and Technology, Japan for Scientific Research (C) (20580013, 2008-2010) and by a fellowship to N. K. from the Japan Society for the Promotion of Science. MALDI-TOF/MS analysis was supported by the Japan Advanced Plant Science Network.

The authors have declared no conflict of interest.

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- 544

Figure legend

Figure 1. 13S globulin α polypeptides detected by CBB staining and the anti-glutelin and anti-glycinin antibodies.

Globulin fractions extracted from common buckwheat flour (var. Shinano-ichigo) (CB) and tartary buckwheat seeds (var. FT Rotunadation) (TB) were electrophoresed and compared by CBB staining (B) and western blotting with the anti-glycinin (C) and anti-glutelin (D) antibodies. The detected band patterns were schematically shown (A). Major bands were tentatively named $\alpha 2$ - $\alpha 10$ for common buckwheat and αx , $\alpha 1$, $\alpha 6$ - $\alpha 10$ for tartary buckwheat. Approximate molecular sizes (kDa) are shown on the left side of panel B. The position of 8S globulin and 13S β polypeptide was also indicated.

Figure 2. Separation and classification of the 13S globulin α polypeptides by 2D-PAGE with the anti-glutelin and anti-glycinin antibodies.

Globulin fractions of common buckwheat flour (var. Shinano-ichigo) were separated by 2D-PAGE and detected by CBB staining (A), silver staining (B), and with the anti-glycinin (C) and anti-glutelin (D). 13S globulin α polypeptides migrated into the left side (acidic side, enclosed by square) in panel A and into the center in panels B-D. Samples prior to IEF separation were also applied to the left side end in panels C and D. The tentative names for the major bands in Figure 1 were matched to the series of streaking spots in panel B. IEF was conducted with IPG strips (pH3-10 for panel A and pH4-7 for panels B-D). Left: acidic; right: basic.

Figure 3. Schematic representation of the 13S globulin subunits.

Primary structures of the known 13S globulin subunits (pro-form, α and β polypeptide with signal peptide) of common buckwheat were compared (GenBank accession No. D87980, AF152003, D87982, AY256960). The subunits are classified as Met-poor and Met-rich according to the methionine content of each subunit. The Met-poor subunits have tandem repeats with different lengths (shown as a number in brackets with the α polypeptide). Methionine (Met) and cysteine (Cys) contents are shown as molar ratios. Calculated molecular size was indicated for each polypeptide. Horizontal arrows denote the position for PCR primers.

Figure 4. Relationship between the PCR amplified bands and protein bands for the 13S globulin α polypeptides at a single seed level.

Crude genomic DNA and globulin fractions extracted from 8 and additional 29 single seeds (common buckwheat var. Shinano-ichigo) were individually analyzed by PCR (panels A and C), CBB staining (panels B and D), and western blotting with anti-glycinin (E) and sequential use of anti-glutelin after anti-glycinin (F). PCR bands amplified for the tandem repeat region of the Met-poor subunits were named mp2-mp8 (panel A). The number in the parentheses of PCR bands indicates the number of tandem repeats. Protein bands (a2-a4) designated in Figure 1 are also indicated. The number under the figures shows individual seed number. Horizontal arrows indicate possible association between PCR and SDS-PAGE bands.

Figure 5. Deduced amino acid sequences and hydrophobicities over the tandem repeat inserts of the Met-poor subunits.

The amino acid sequences deduced from the PCR bands amplified for the Met-poor subunits (mp2-mp8, in Fig. 4A) were compared with that of D87980, AF152003, and D87982. The amino acids spanning 108-212 residues (D87980) from the N-terminus of the mature α polypeptides were aligned. The hydrophobicity profile of D87980 (full length) was examined by a technique used by Kyte and Doolittle (1982). The horizontal double-headed arrow indicates the position of the tandem repeat insert (5 times).

Figure 6. Digestibility of the 13S globulin α polypeptides by trypsin.

Globulin fractions extracted from the buckwheat flour ‘Shinano-ichigo’ were incubated with 1/100 (w/w) trypsin for two hours at 37 °C. Aliquots taken at the indicated time were electrophoresed and compared by CBB staining (A) and western blotting with the anti-glutelin antibody (B). The number in brackets after the major band names (a2-a10) indicates the possible tandem repeat lengths. The number below the figure shows the incubation time (minutes). The position of 8S globulin, 13S α and β polypeptides was also indicated.

607 **Highlights**

- 608 ● β polypeptides of 13S globulin subunits are prevalent allergen in common buckwheat.
- 609 ● α polypeptides, a counterpart of the β polypeptides, were minutely characterized.
- 610 ● Four new methionine-poor subunits with 0, 2, 4, 6 tandem repeats were identified.
- 611 ● The insertion of the tandem repeats enlarged the diversity of the α polypeptides.
- 612 ● The insertion also reduced the stability of the α polypeptides against trypsin.

613

614

Figure 1

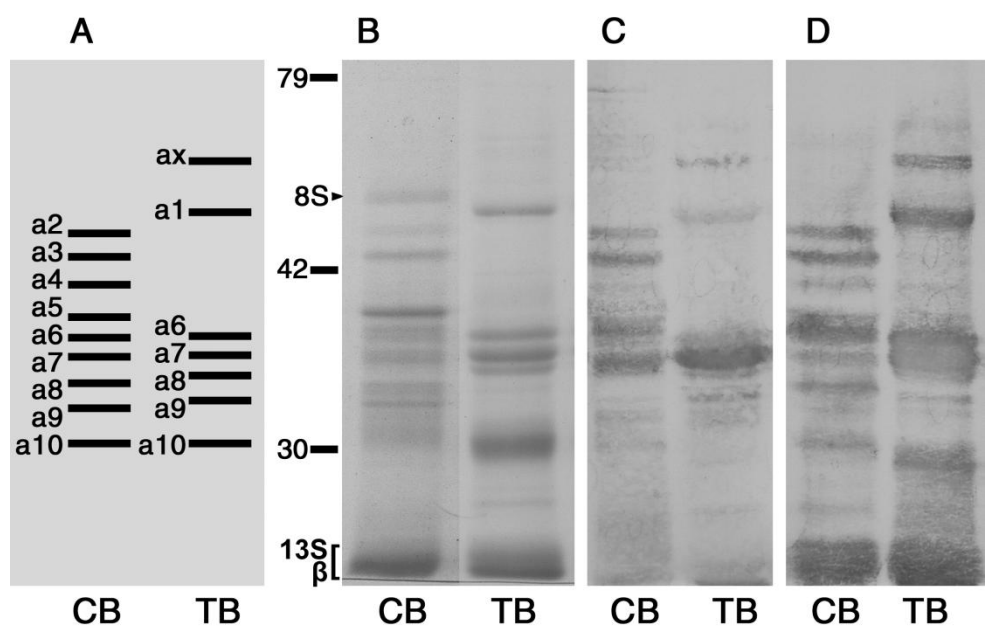


Fig. 1

Figure 2

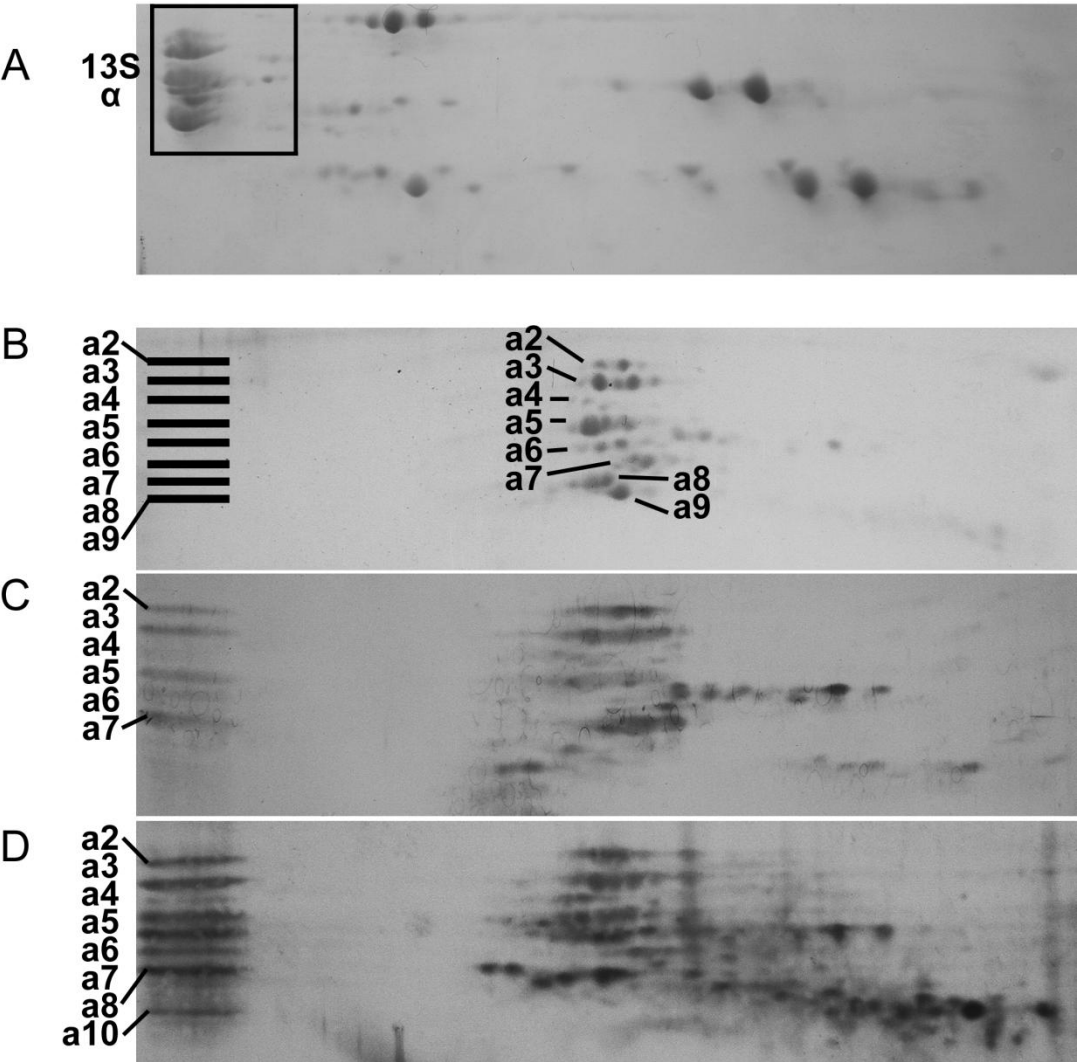
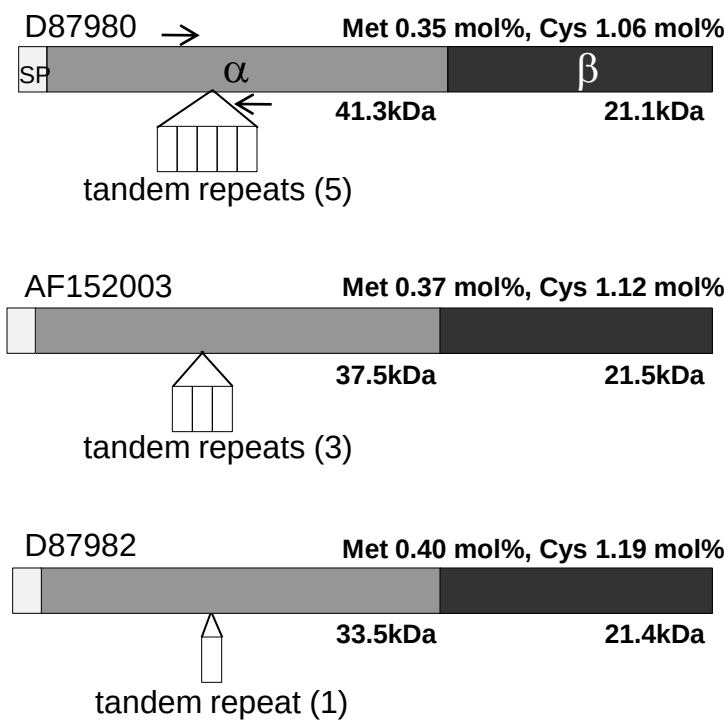


Fig. 2

Figure 3

Met-poor subunit



Met-rich subunit

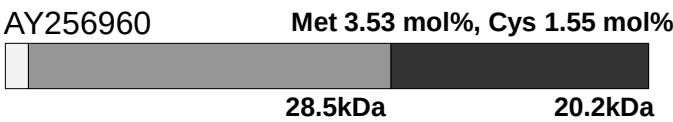


Fig. 3

Figure 4

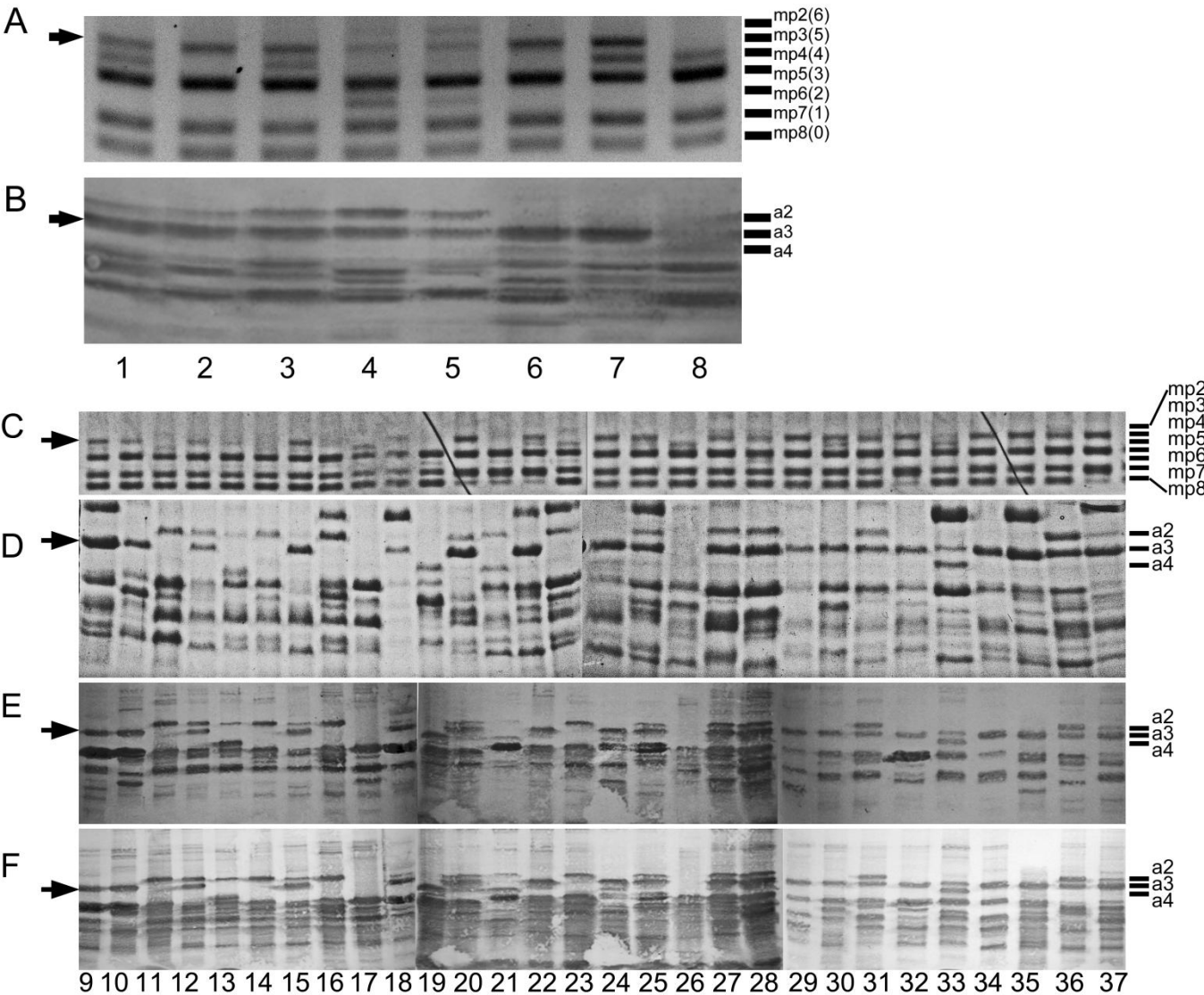


Fig. 4

Figure 5

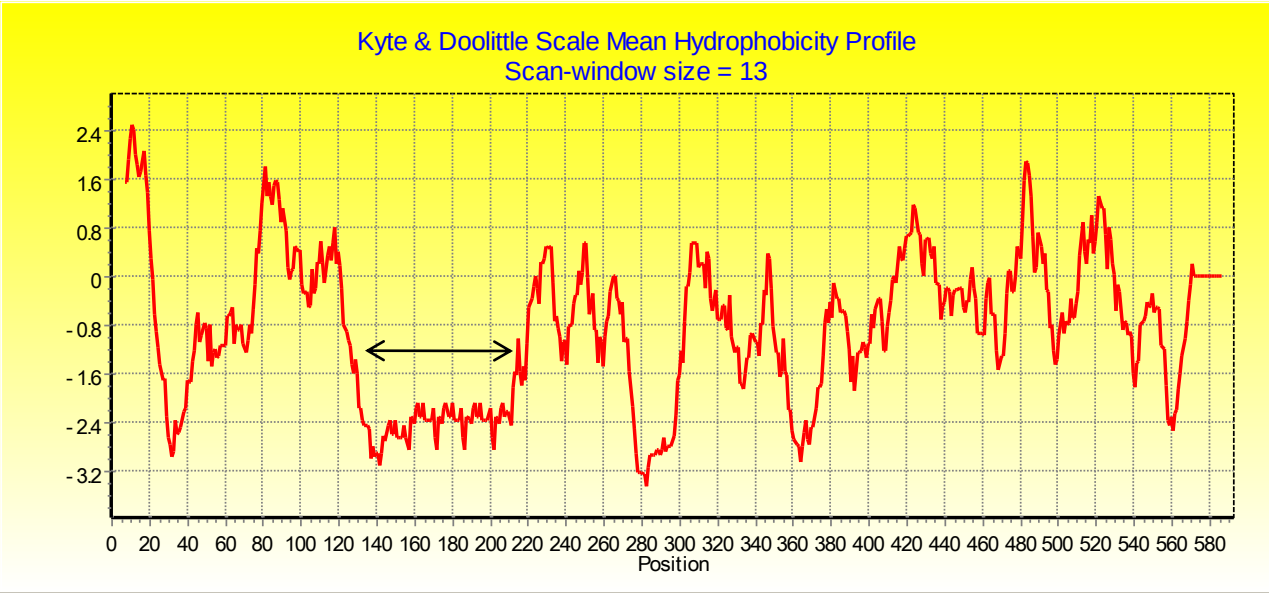
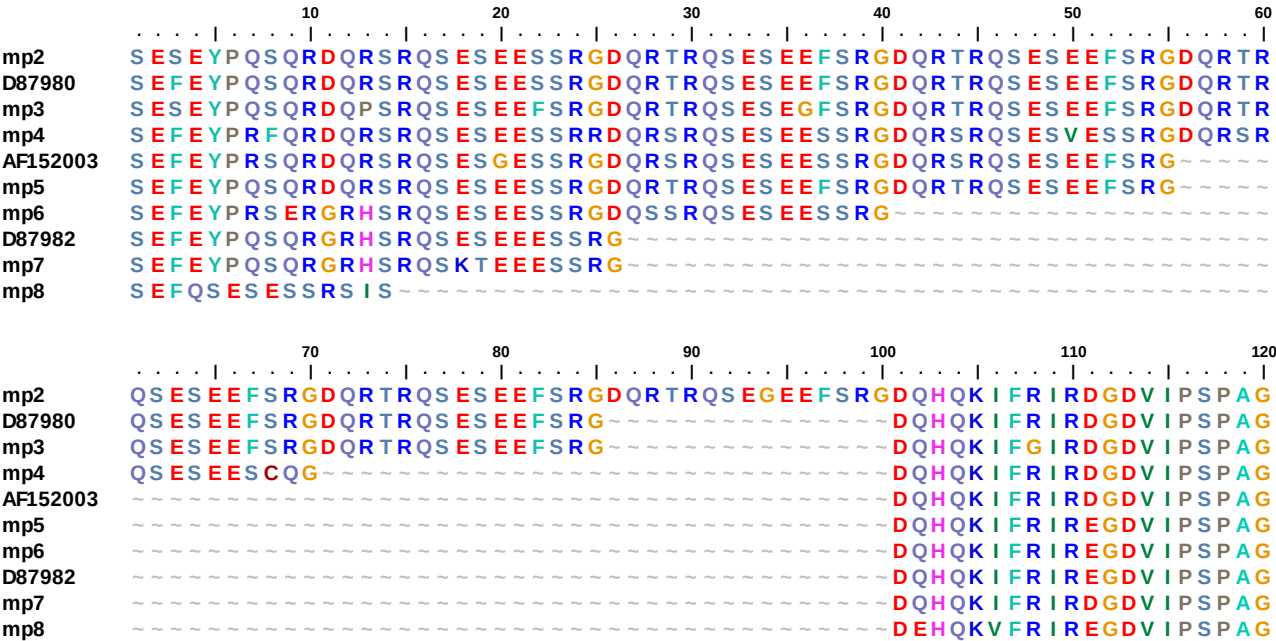


Fig. 5

Figure 6

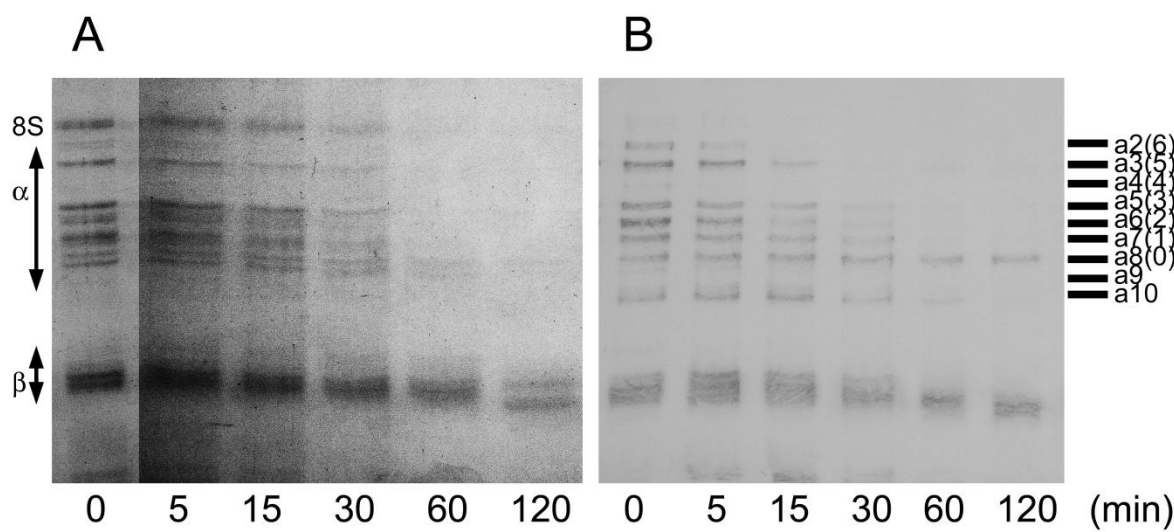


Fig. 6