# Analysis of the Major Seed Storage Protein, 13S Globulin, in Common Buckwheat (*Fagopyrum esculentum* Moench)

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**Abstract**

Common buckwheat seed proteins are valuable proteins with high nutritional and biological values. The proteins are also health-promoting proteins with many physiological functions, such as the ability to lower blood cholesterol and to prevent accumulation of fat, constipation, mammary carcinogenesis and colon carcinogenesis. However, the proteins are allergenic to human beings. The elimination or mitigation of the allergenic proteins is highly desired for the enhancement and potential utilization of buckwheat as a food crop. The prevalent allergen of common buckwheat, Fag e 1, 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 in this study to better understand this allergenic protein. The 13S globulin α polypeptides were categorized into three types and were further grouped into methionine-poor and methionine-rich subunits as major and minor types, respectively. Besides the three known methionine-poor subunits, four new methionine-poor subunits with 0, 2, 4, and 6 tandem repeat inserts were identified. Highly polymorphic band pattern and its correlation between SDS-PAGE and PCR analyses at a single seed level suggested that the large variation among the α polypeptides was explained by the different lengths of tandem repeat inserts. In agreement with the fact that the tandem repeat region is hydrophilic with many arginine residues, digestibility against trypsin, that is one of critical characteristics for food allergen, was different between the subunits with and without tandem repeat inserts.

*Keywords: 13S globulin, seed storage protein, common buckwheat (*Fagopyrum esculentum *Moench)*

## Introduction

The allergens of buckwheat seeds have been identified and characterized 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 least prevalent allergens. 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. The Fag e 1-null mutant has been sought, but it has not been discovered. However, the content of a Fag e 1 has been demonstrated to change among cultivars (Maruyama-Funatsuki *et al.*, 2004).

Buckwheat seeds contain 8.5% to 18.9% protein. The most abundant protein is the 13S globulin, which is salt-soluble and accounts for approximately 43% of the total seed protein. The 13S globulin is a storage protein and resembles the legumin-like seed storage protein of other species, such as rice glutelin and soybean glycinin. It is considered a member of the 11S globulin family based on its sedimentation constant, amino acid homology and similarities in biosynthetic and accumulation processes. Like other legumin-like, seed storage proteins (i.e., rice glutelin and soybean glycinin), 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.

The subunits of seed storage proteins show different characteristics from each other. However, the subunit composition of buckwheat has seldom been of concern, except in some studies by Cepkova & Dvoracek (2006), Rogl & Javornik (1996), and Bonafaccia *et al.* (1994). In fact, even the differences in the subunit structures and the maximum number of subunit types are not well known. 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 and 2D-PAGE, coupled with immunodetection using two unique antibodies against the 13S globulin-related rice glutelin and soybean glycinin. Four new subunits with various lengths of tandem repeats were confirmed by PCR analysis at a highly polymorphic single seed level. By showing different digestibilities among the subunits, we proposed the possibility to develop novel buckwheat plants with lowered allergenicity.

## Materials and Methods

### 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 a single seed flour or commercial flour 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) for 2D-PAGE analysis.

### SDS-PAGE, 2D-PAGE, and Western blot Analyses

SDS-PAGE was performed using 14%T acrylamide gel at a constant voltage of 200 V. Isoelectric Focusing (IEF) was performed with IPG strips pH range 4-7 (BioRad) 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 min, 400 V for 10 min, 1000 V for 10 min, and 1500 V for 12 hrs. The immunoblotting was performed according to the method mentioned in our previous report (Khan *et al.*, 2008). Anti-rice glutelin and anti-soybean glycinin antibodies (Katsube-Tanaka *et al.*, 2004; Katsube *et al.*, 1999) were used to detect the 13S globulin α polypeptides of common buckwheat.

### 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), AGGATG(C/T)CCGGAGAC(A/G)T(A/T)CCA and reverse (right1), CTAACGTTC(C/T)CATCGAGCTG for the Met-poor subunits.

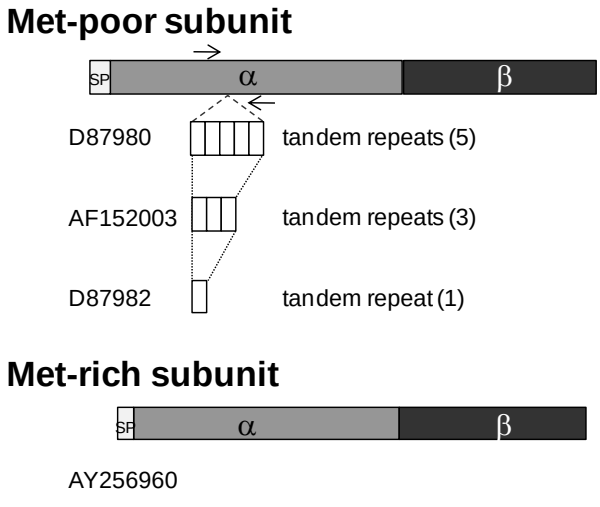
### Trypsin digestion

Globulin fractions extracted from the 'Shinano-ichigo' buckwheat flour were incubated with 1/100 (w/w) trypsin (Promega, sequence grade) for two hours at 37°C according to the manufacturer's instruction. Aliquots taken at a given times were electrophoresed and compared by CBB staining and western blotting with the anti-glutelin antibody.

**Results and Discussion**

The 13S globulin fractions from commercial flour of common buckwheat and tartary buckwheat seeds were resolved by SDS-PAGE, and detected by immunodetection using anti-glycinin and anti-glutelin antibodies. 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) (data not shown). The α polypeptides of common buckwheat were ranged from 30 to 47 kDa according to the mobility in SDS-PAGE.

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 2D-PAGE using anti-glycinin and anti-glutelin antibodies. The major 8 bands recorded on SDS-PAGE gels were resolved into several distinct spots with different pI values and with slightly different molecular weights on 2D-PAGE gels, forming a horizontal streak (data not shown). Each horizontal streak of spots was collectively named, instead of naming each spot individually in this study. When the spots were examined with the anti-glycinin and anti-glutelin antibodies, most spots reacted against either of two antibodies with different degrees except the spot a9.




| | Mol% | | kDa | | pI | |
|---|---|---|---|---|---|---|
| | Met | Cys | α | β | α | β |
| D87980 | 0.35 | 1.06 | 41.3 | 21.1 | 5.2 | 9.1 |
| AF152003 | 0.37 | 1.12 | 37.5 | 21.5 | 5.4 | 9.2 |
| D87982 | 0.40 | 1.19 | 33.5 | 21.4 | 5.2 | 9.7 |
| AY256960 | 3.53 | 1.55 | 28.5 | 20.2 | 5.0 | 6.7 |

Figure 1. 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). Horizontal arrows denote the position for PCR primers.

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. 1). The three Met-poor subunits contain variable tandem repeat sequences. For example, 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. 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. 1) over the region containing the tandem repeats of the Met-poor subunit to explore novel genes. The PCR amplification with the crude genomic DNA produced seven types of bands, which were named mp2-mp8 (data not shown). 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.

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 (data not shown). In addition, the mp2-mp8 bands and a2-a8 bands were observed at equally spaced intervals (data not shown). 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 amino acid sequences deduced from PCR amplified bands mp2-mp8 were compared with those of D87980, AF152003, and D87982 (Fig. 2). The alignment clearly showed the tandem repeat regions had less diversity, except the lengths of the bands. However, it is notable that the tandem repeat regions and their vicinities had many arginine residues, and the regions were hydrophilic according to a method used by Kyte & Doolittle (1982), 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.

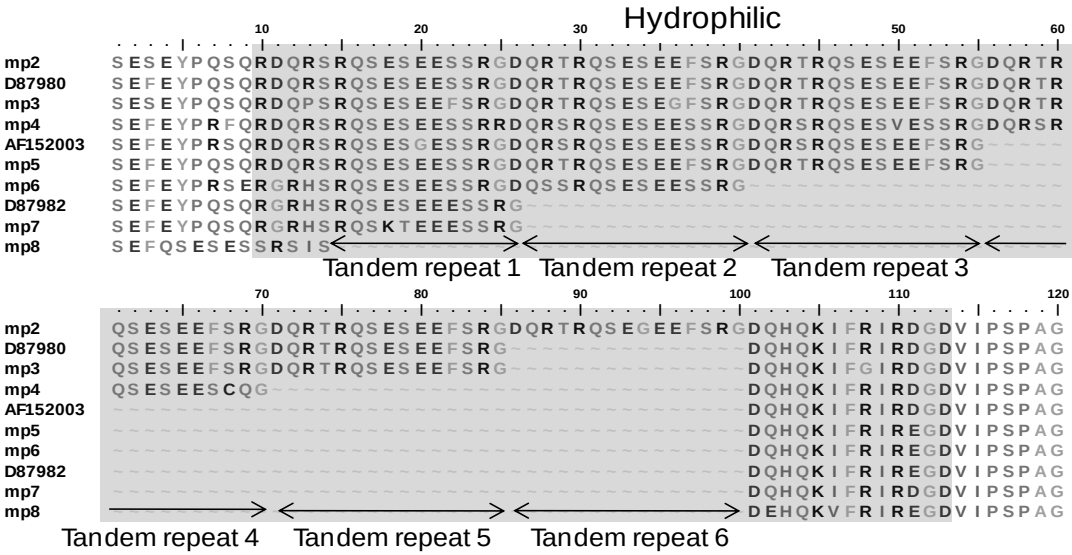



Figure 2. Deduced amino acid sequences and hydrophobicities over the tandem repeat inserts of the Met-poor subunits.

The amino acid sequences deduced from the PCR bands amplified for the Met-poor subunits (mp2-mp8) were compared with that of D87980, AF152003, and D87982. The amino acids spanning 108-212 residues from the N-terminus of the mature α polypeptides (D87980) were aligned. The position of tandem repeats 1-6 and approximate hydrophilic area were shown by double-headed arrows and gray color shades, respectively.

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 (data not shown). 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, which

seems to be derived from a Met-rich subunit having relatively higher cysteine contents, was rather resistant to digestion according to the CBB staining (data not shown).

It has been described that resistance to digestion and processing is one of the more important characteristics for food allergenic proteins (Bannon, 2004). Sen *et al.* (2002) showed peanut allergenic protein stability upon proteinase digestion affected IgE-binding epitopes intactness. Even though the difference in the digestibility of the β polypeptides was empirically not revealed in this study, 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 the hypothesis is correct, the Met-poor subunits of the 13S globulin may have different digestibilities and distinct potential allergenicities. Although our preliminary analysis on pepsin digestibility demonstrated little difference among the subunits (data not shown), 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' sera should be required.

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