The digestibility of proteins in red kidney bean (*Phaseolus vulgaris* L.) was examined by *in vitro* pepsin assay. A 20-kDa polypeptide that remained highly stable after heat processing was identified as a basic subunit of legumin. The results of a monobromobimane (mBBr) labeling test implied that this protein contained rigid intramolecular disulfide bonds, which might contribute to pepsin resistance.

**Key words:** pepsin-resistant protein; red kidney bean; legumin

Plant foods are critical resources for global human nutrition, supplying about 65% of food proteins. Among a variety of plant foods, beans are low in fat, low in sodium, high in protein, and a good source of fiber, certain minerals, vitamins, and anti-oxidant polyphenols. But bean proteins in general have lower nutritional value due to a deficiency in one or more essential amino acids and lower digestibility. The digestibility of protein causes another concern in respect of risk of allergenicity. Astwood *et al.* suggested that stability to pepsin digestion is a significant and valid parameter that distinguishes food allergens from non-allergens. In this study, the protein quality of red kidney bean, one of the representative varieties of common beans in Japan, was assessed by *in vitro* pepsin digestion.

Flour of dehulled red kidney bean (*Phaseolus vulgaris* cv. Taishokintoki) was supplied by the Bean Fund Association of Japan. Heat-processed bean paste was prepared by mixing 10 g of flour with 30 ml of water in a sealed plastic bag, followed by boiling for 10 min. Distilled water was added 10 times (v/w) to flour and 2.5 times (v/w) to paste, then homogenized with a Hiscotron (NS-50, Nichi-On, Tokyo) for 1 min at 10,000 rpm. After centrifugation at 8,000 × g for 20 min, supernatants were collected. The protein concentration of the extracts was estimated by the micro assay procedure for a protein assay reagent (BioRad, Tokyo).

For complete reduction, a sample was boiled for 5 min in 5 mM DTT. Reduced samples were mixed with 5 μl of 20 mM monobromobimane (mBBr) and incubated for 15 min. The reaction
was completed with 10 μl of 100 mM β-mercaptoethanol and 5 μl of 20% SDS, and then 10 μl of 80% glycerol containing 0.005% bromophenol blue was added to prepare for SDS–PAGE. Samples without reduction treatment were also labeled by mBBr as a control. Electrophoresis was carried out as described above. Gels were soaked in 12% of trichloroacetic acid for 1 h and washed with 40% methanol and 10% acetic acid for 2 h. The mBBr-labeled proteins were detected under 365 nm UV light.

The pepsin digestibility of intact and that of heat-processed red kidney bean is compared in Fig. 1. In the intact flour, phaseolin, the major protein, was highly stable to pepsin digestion, but heat processing with water remarkably improved its digestibility. Deshpande and Nielsen have reported that heated phaseolin was highly susceptible to a number of proteolytic enzymes in vitro. A decrease in phaseolin in the cooked paste implied that partial degradation of phaseolin might occur during heat processing. A 20 kDa polypeptide also appeared to be tolerant to pepsin digestion. In contrast to phaseolin, the 20 kDa polypeptide remained resistant to pepsin digestion after heat processing (Fig. 1B). Lectin (30 kDa) and 51 kDa polypeptide, which were promptly digested in intact flour, showed increased resistance to pepsin in the heat-processed paste.

In order to identify the pepsin-resistant 20 kDa polypeptide, proteins extracted from 2 min of digestion of heat-processed paste was transblotted onto PVDF membrane, and the excised 20 kDa band was subjected to automated polypeptide sequencing. The N-terminal amino acid sequence of the 20 kDa pepsin-resistant protein matched that of the basic subunit of legumin in common bean reported by Muhling et al. (Table 1).

Table 1. N-Terminal Amino Acid Sequences of a Pepsin-Resistant Polypeptide and Two Disulfide Proteins

<table>
<thead>
<tr>
<th>Protein band/spot</th>
<th>Amino acids sequences</th>
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<tbody>
<tr>
<td>20 kDa pepsin restant polypeptide</td>
<td>GIEETL?TLK</td>
</tr>
<tr>
<td>54–45 kDa disulfide protein (a)</td>
<td>TTNLFNRCRI</td>
</tr>
<tr>
<td>20 kDa disulfide protein (b)</td>
<td>GIEETL?TLKQ(H)N?ARA</td>
</tr>
<tr>
<td>Legumin α-subunit*</td>
<td>TTNLFNRCRI</td>
</tr>
<tr>
<td>Legumin β-subunit*</td>
<td>GIEETLCTLQKHNIARASS</td>
</tr>
</tbody>
</table>

?, unidentified amino acids; *M. Muhling, et al.5)

Disulfide protein in red kidney bean was examined by nonreducing/reducing two-dimensional SDS gel electrophoresis (Fig. 2A). In this experiment, polypeptides without disulfide bonds were located on the diagonal line, and polypeptides having only intramolecular disulfides shifted to the upper side of the line. As shown in Fig. 2A, several spots (approximately 60, 35–50, 25, 18, and 10–15 kDa) shifted in second-dimensional electrophoresis under reducing conditions. Meanwhile, protein subunits combined with intermolecular disulfide bonds were detected as spots under the diagonal line. Derived from about 70 kDa protein in first-dimensional electrophoresis, conjugated spots ranged from 54 to 35 kDa, and 20-kDa spots were found as polypeptides with intermolecular disulfide bonds. In the results of N-terminal amino acid analysis, the sequence of 20-kDa polypeptides was identical to that of basic subunits of legumin, while the sequence of 54 to 45 kDa spots coincided with acidic subunits (Table 1). Legumins, a group of seed-storage proteins, are hexameric molecules of average Mr about 360 kDa comprised of six pairs of subunits, acidic subunits of about 40 kDa and basic subunits about 20 kDa. The acidic subunits of red kidney bean ranged from 54 kDa down to about 35 kDa, while the basic subunit appeared to be in a single size class. It is known to be a characteristic to legumin group proteins that basic subunits are highly conserved in size and sequence and that the acidic subunits are much more heterogeneous.7) Muhling et al. found that acidic subunits of Phaseolus legumin different in size shared the same N-terminal sequence.6)
The reactivity of intramolecular disulfide bonds in red kidney bean protein was estimated by the mBBr labeling test (Fig. 2B). Samples were incubated under various reducing conditions, and then the reduced (–SH) forms of target proteins were derivatized with mBBr. After subsequent separation by SDS–PAGE, disulfide proteins appeared as fluorescent bands viewed under ultraviolet light. Four polypeptides (62, 47, 16, and 15 kDa) were detected as major disulfide proteins in the samples moderately reduced by DTT, DTT-reduced thioredoxin, or the NADPH-thioredoxin system (lanes 3, 4, and 7). Several minor bands, including a 25 kDa band, were also detected under moderate reduction. These results support the shifting of intramolecular disulfide proteins shown in Fig. 2A. Two additional polypeptides (70 and 20 kDa) were detected in the completely reduced sample (lane 2), implying that they contained one or more rigid disulfide bonds in the molecule. The 20-kDa polypeptide is the basic subunit of legumin described above. The 70-kDa polypeptide has not yet been identified. In our previous work, it was found that disulfide bonds in two major rice allergens, α-globulin and α-amylase trypsin inhibitor, were hardly reduced by moderate reduction, similarly to these bean polypeptides. It has been reported that a buried, rigid disulfide (Cys106–Cys119) maintains the conformation of β-lactoglobulin, a major milk allergen.

In this study, it was found that a 20-kDa polypeptide in red kidney bean, identified as a basic subunit of legumin, was remarkably tolerant of pepsin digestion. Digestibility by pepsin is thought to be a parameter of the allergenicity of unknown proteins. So far, it has not been elucidated whether legumin, a minor protein component in red kidney bean, is involved in bean allergy. But we should be cautious because allergenicity and cross reactivity have been reported for some legumin-type proteins in plant seeds, such as buckwheat legumin and soybean glycinin. Further analysis of the sequence and structure of bean legumin should reveal its allergenic properties by the bioinformatics method of allergenicity assessment.

The 20-kDa polypeptide appeared to have rigid intramolecular disulfide bonds, which may contribute to tolerance to proteolysis. De Val et al. suggested that controlling specific disulfide bonds in protein can lower the allergenicity of milk. Quality evaluation of legumin proteins in common beans and controlling rigid disulfides in food proteins are necessary to make best use of this plant resource in our daily diet and to achieve food safety.

Acknowledgment

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References


