Occurrence of Astringent Oligomeric Proanthocyanidins in Legume Seeds*

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The distribution of oligomeric proanthocyanidins in legume seeds was investigated in connection with organoleptic astringency. The seeds contained various kinds of oligomers (dimers~hexamers). The total oligomer contents were at least 0.040% in azuki beans, 0.017% in black soybeans, 0.005% in mung beans and a trace amount in regular soybeans. Each aliquot of aqueous solution of partially purified proanthocyanidin oligomers tasted astringent even at a concentration of 0.001% (w/v). The possible contribution of oligomeric proanthocyanidins and polymeric proanthocyanidins to the astringent off-taste of legume seeds is discussed.

Proteins in legume seeds have not yet been utilized partly because of their astringent off-taste. No satisfactory explanation for this off-taste, however, has been given and no effective technique for removing the organoleptic astringency from legume seeds has been developed until now.

On the other hand, several investigations on astringent components in fruits and their products, such as avocado fruits and apples, have been carried out during the last twenty years. Geissman and Dittmar¹⁾ revealed that dimeric proanthocyanidins isolated from avocado fruits tasted astringent. Lea²⁾ reported that higher-molecular-weight procyanidins contributed to the astringency of cider.

Recently, some workers reported the occurrence of condensed tannins in some legume seeds. Martin-Tanguy *et al.*³⁾ reported that horse bean seed-coats contain proanthocyanidin-type tannins. Price *et al.*⁴⁾ measured the condensed flavan contents of some legume seeds from the view point of their nutritional effects. Until now, however, there has been no paper reporting the occurrence of

oligomeric proanthocyanidins in legume seeds. And no report has described the contribution of oligomeric and polymeric proanthocyanidins to the astringent off-taste of legume seeds.

This paper describes the detection of proanthocyanidins in legume seeds, the determination of anthocyanidin formation, and the strength of organoleptic astringency of partially purified proanthocyanidins.

MATERIALS AND METHODS

Raw materials. Azuki beans (*Vigna angularis* Ohwi et Ohashi, var. Dainagon), black soybeans (*Glycine max* L., Hikarikuro), soybeans (*Glycine max* L., an American cultivar) and mung beans (*Vigna radiata* Wilcz., from Indonesia) were commercially purchased.

Extraction and fractionation of proanthocyanidins. These beans were freeze-dried, powdered in a Waring blender and passed through a 12 mesh sieve. After being defatted with petroleum ether and acetone at 20°C, each sample was dispersed in 70% aqueous acetone containing 0.1% (w/v) ascorbic acid for 20 hr at 20°C. The mixture was filtered and the residue was washed with 70% aqueous acetone. The filtrates and washings were combined and saturated with NaCl to salt out the acetone.⁵⁾ By washing the acetone phase again with water saturated with NaCl and acetone the crude extract was obtained. The acetone was removed *in vacuo* from the extract thus obtained. The residue was dissolved again in an equal volume of water.

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The aqueous solution was washed three times with diethyl ether. The aqueous phase was then extracted with an equal volume of ethyl acetate three times (Fraction-I; F-I). The aqueous phase thus obtained was then saturated with NaCl and reextracted with an equal volume of ethyl acetate (F-II). The residual aqueous phase was then extracted with an equal volume of acetone (F-III).

Partial purification of proanthocyanidins. Proanthocyanidins in F-I, F-II, and F-III were partially purified by Sephadex LH-20 chromatography (column size; 2.5×39 cm).⁶⁾ After washing the Sephadex column successively with 5% EtOH and 20% MeOH, proanthocyanidins were eluted with 60% acetone and proanthocyanidin fraction-I (PF-I), PF-II, and PF-III were obtained, respectively.

Isolation and detection of proanthocyanidins. Proanthocyanidins in PF-I were separated and detected by two-dimensional thin-layer chromatography (TLC) in the same way, as reported by Van Buren *et al.*⁷⁾ Proanthocyanidin oligomers in PF-II were separated and detected by silica-gel HPTLC according to the method of Lea.²⁾ Proanthocyanidin polymers in PF-III were separated from each other by Sephadex LH-20 gel filtration according to the method of Strumeyer and Malin.⁸⁾

Methods of analysis. The conversion of leucoanthocyanin to anthocyanidin,⁹⁾ the controlled-hydrolysis¹⁰⁾ of proanthocyanidin, the identification of acid-hydrolysates¹⁰⁾ and the determination of total flavans¹¹⁾ were carried out in the same ways as described in the references.

Sensory evaluation test. The isolated proanthocyanidins were dissolved in pure water to prepare 0.0100%, 0.0010% and 0.0001% solutions. And 3.0 ml aliquots of the solutions were tasted by a panel composed of five judges who were selected for their high sensitivities to organoleptic astringency.

RESULTS AND DISCUSSION

Roux¹²⁾ reported that leucoanthocyanidins (monomeric proanthocyanidins: MW = 290 ~ 322) shows no significant tanning properties. Bate-Smith¹³⁾ showed that these oligomers have tanning properties, and that the measure of relative astringency increases in the order of dimers, trimers and tetramers. Generally, the tanning action of polyphenols considerably depends on their molecular size and it was shown that the maximum tanning action, in other words, maximum astringency, is demonstrated by polyphenols of inter-

mediate size (a molecular weight range of 500 ~ 3000).¹⁴⁾ Consequently, it seems important to investigate the molecular size of proanthocyanidins in order to discuss the organoleptic astringent taste of them.

Until now, however, it has been unknown whether proanthocyanidins of intermediate size are present in legume seeds or not. Therefore, at first we tried to examine the occurrence of oligomeric proanthocyanidins in some legume seeds.

It was difficult to detect oligomeric proanthocyanidins directly in the crude extracts from beans because of their lower concentrations in comparison with those of monomeric, polymeric flavan pigments, *etc.*

FIG. 1. Cellulose Thin-layer Chromatograms of PF-Is from Legume Seeds.

Detection reagent: vanillin-HCl. (A), azuki beans; (B), black soybeans; (C), mung beans; (D), soybeans.

FIG. 2. Silica HPTLC of PF-IIIs from Legume Seeds.

Solvent: toluene-acetone-formic acid (3:6:1, v/v/v). Numerals indicate degree of polymerization: S, standards from cider²⁾; A, azuki beans; B, black soybeans; C, mung beans; D, soybeans.

FIG. 3. Gel Filtration of PF-III from Azuki Beans on Sephadex LH-20 with 50% Aqueous Acetone.

Consequently, proanthocyanidins in the crude extract were preliminarily fractionated into three fractions, F-I, F-II, and F-III, in order of molecular weight. They were further purified by means of Sephadex LH-20 chromatography, and PF-I, PF-II, and PF-III were obtained, respectively. These PFs contained 50~88% flavans.

The HPTLC of PF-Is indicated that they were mainly composed of dimers. Since proanthocyanidins usually have many isomers, PF-Is from four kinds of beans were examined by Avicel SF cellulose TLC (Fig. 1). Actually, several spots of lower oligomers were detected for all the kind of beans. Figure 2 shows the HPTLC of PF-IIIs. The results indicated the occurrence of oligomers (trimers~hexamers) in azuki beans and black soybeans. To our knowledge, this is the first report of the occurrence of oligomeric proanthocyanidins in legume seeds. The novel technique (ethyl acetate extraction after saturating aqueous phase with NaCl) was found to be very effective for the isolation of oligomers.

The HPTLC of PF-IIIs indicated that a large part of PF-IIIs is composed of polymeric flavans (proanthocyanidin type). Actually, gel filtration of PF-IIIs indicated the occurrence of many kinds of polymeric proanthocyanidins of various molecular weights in all the sorts of beans. For instance, Fig. 3 shows a gel chromatogram of PF-III from azuki beans.

The crude extract from each bean produced

TABLE I. ANTHOCYANIDIN FORMATION (mg)^a

Fraction	Azuki beans	Black soybeans	Mung beans	Soybeans
F-I	12.0	5.1	1.1	>0
F-II	28.2	11.8	4.0	>0
F-III	78.5	23.6	59.8	>0.14

^a Results are recorded as values per 100 g of beans.

anthocyanidins ($\lambda_{\max} = 554 \text{ nm}$ in 5% HCl in *n*-BuOH) on acid hydrolysis. Table I shows anthocyanidin formation¹⁵⁾ from F-Is, F-IIIs, and F-IIIs through acid hydrolysis. These values were supposed to indicate minimum contents of proanthocyanidins in these legume seeds. These results indicated that the oligomeric proanthocyanidin content decreases in the order of azuki beans, black soybeans, mung beans, and regular soybeans. On the other hand, the polymeric proanthocyanidin content decreases in the order of azuki beans, mung beans, black soybeans and regular soybeans. In particular, it is noteworthy that the proanthocyanidin content of black soybeans is much higher than that of regular soybeans. This result corresponds to the fact that black soybeans taste much more astringent in comparison with regular soybeans.

Treatment of each PF (PF-Is, PF-IIIs, and PF-IIIs) other than that from soybeans with 0.1 N-hydrochloric acid in ethanol at room

temperature yielded (–)epi-catechin and unknown products on the paper chromatogram. Treatment with 5N-hydrochloric acid in ethanol at 60°C gave cyanidin and unknown products. As for the PFs from soybeans, these phenomena could not be confirmed because of the shortage of samples due to the low content of these substances.

Aqueous solutions of each PF other than that from soybeans tasted astringent even at a concentration of 0.0010% (w/v). As for the organoleptic taste of PFs from soybeans, it was impossible to evaluate it for the same reason mentioned above. These results suggested that these PFs may taste more astringent than any other natural compounds reported.¹⁶⁾

PF-IIIs as well as PF-Is and PF-IIs showed significant organoleptic astringency. However, aqueous solutions of highly polymeric proanthocyanidins such as peak-1 and peak-2 (Fig. 3), which contained no proteinaceous constituents, did not taste astringent at the concentration of 0.0100%. This result suggests that the PF-III from azuki beans contains both non-astringent proanthocyanidins (higher polymers) and astringent proanthocyanidins (lower polymers).

In conclusion, oligomeric and low polymeric proanthocyanidins may perhaps contribute to the astringent off-taste of legume seeds such as azuki beans, black soybeans, and mung beans.

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