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Biochemical Characterization of Building Block of Condensed Tannin in Faba Bean (*Vicia faba* L.)

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Abstract
Despite being nutritionally rich, faba bean is not considered as commercial legume crop. One of the probable reasons for it, is the lesser acceptance due to high content of condensed tannins in seed coat. Condensed tannins are polymer of flavan-3-ols type monomers like catechin and/or epicatechin. The knowledge about these monomers is highly important to design strategies to modulate its content in target parts of the plant. In this study we first confirmed the presence of condensed tannin in seed and further its localization in seed coat by tannin specific reagent. The enzyme responsible for biosynthesis of catechin/epicatechin i.e. antocyanidin reductase, was purified from leaf extract. We further demonstrated the presence of epicatechin as monomer in seed extract of faba bean which was further confirmed by *in-vitro* anthocyanidin reductase catalyzed reaction using paper chromatography. The enzyme and/or gene responsible to synthesize epicatechin can now be an ideal target for reducing the amount of condensed tannin in seed coat, and hence can be useful for nutritional improvement of faba bean

Keywords
proanthocynidin, flavan-3-ols, catechin, epicatechin, anti-nutritional factor

Introduction

Faba bean as a plant food for human consumption provides a balanced diet of lysine-rich protein, carbohydrates, fibre, and good source of iron, magnesium, potassium, zinc, copper, selenium, and many vitamins (Friedman 1996, Kumar *et al*. 2015, Sinha, 2014). Furthermore, faba bean has also been attributed with its certain medicinal values. For instance, faba bean is a source of levodopa, a natural precursor of dopamine which has potential use in the treatment in Parkinson's disease. Levodopa was identified in the seedlings, pods and beans of the faba bean (*Vicia faba*) by Guggenheim (1913). Faba bean is often grown as vegetable crop in India. Currently it is gaining importance as a pulse crop in the cropping pattern of north Bihar, eastern UP and MP. Its yield potential has already been realized by considerable number of reports came from different parts of the India i.e. 2799-4684 kg/ha under irrigated condition in Haryana (Rao *et al*. 1984), 2327-2458 kg/ha in Punjab (Dhingra *et al*. 1990). This pulse crop has been cultivated and sold in a limited scale and quantities to local markets and thus it has never been considered as a commercial pulse crop. A drawback to the more widespread use of the faba bean stems from its toxicity because it contains some antinutritional factors (ANFs). To enhance its market expansion this crops must be accepted by larger population.

Condensed tannins (CTs) are one the most important ANFs located in the testa, which negatively affect their digestibility and other nutritive quality. However, the antioxidant properties of the flavonoids and the phenolics present in different parts of this plant have also gained attention recently (Sinha *et al*. 2013) as far as their protective roles in various stressed conditions are concerned. The flavan-3-ols are the monomers of condensed tannins (CTs, also known as proanthocyanidins PAs), which are widely found in seed coats, leaves, flowers, stems and other tissues throughout the plant kingdom. CTs share the same upstream biosynthestic pathway as the anthocyanin flower pigment (Xie *et al*. 2004). The building blocks or starter units of most PAs are the flavan-3-ols e.g. catechin and epicatechin, whose last common biosynthetic intermediate is leucoanthocyanidin (an intermediate in anthocyanin biosynthesis also referred to as flavan 3, 4-diol), which results from the reduction of flavonols by dihydroflavonol 4-reductase (DFR). Catechin is derived from the reduction of leucoanthocyanidin through the activity of leucoanthocyanidin reductase (LAR) whereas epicatechin formation occurs via anthocyanidin synthesis and reduction, two steps catalyzed by anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR), encoded by leucoanthocyanidin dioxygenase (LDOX)/ ANS and BANYULS (BAN)/ANR genes, respectively (Xie *et al*. 2003). Anthocyanidins are consequently essential for PA biosynthesis in species that accumulate only epicatechin-based PAs. Chemical characterization of the reaction products of ANR identified two isomers of flavan-3-ol, namely 2R, 3R-2, 3-cis and 2S, 3R-2, 3-trans. With cyanidin as substrate, these products are (-)-epicatechin and (-)-catechin, respectively. The 2R, 3R-2, 3-cis-flavan-3-ol, which was the major reaction product, is the common building block of CTs in many plants including Arabidopsis and alfalfa (*Medicago sativa*) (Xie *et al*. 2004). To address the issue of CTs as antinutritional factor in faba bean it is highly important

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to know the building block of CTs in faba bean. Therefore in this study we tried to identify the building block of CT in seed extract of faba bean.

Material and Methods

The faba bean genotype i.e. RAU-1, (Sinha *et al*. 2014) was used in the present study.

Staining of whole seed and seed coat

For staining of either whole seed or only seed coat, the DMACA (p-dimethyl aminocinamaldehyde) reagent in concentration of 2% w/v DMACA in 3 M HCl was used. The duration of staining was initially done at different time interval until it stains whole seed coat and for determination of tannin deposition in seed coat the duration was kept for same time period which it took to stain the seeds completely.

Extraction of Anthocyanidin reductase (ANR)

The enzyme was extracted using the method described by Dellus *et al*. (1997) with few modifications. 200 mg leaf tissue was ground with the help of liquid N_2 and then mixed with a mixture containing 200 mg PVPP (Sigma-Aldrich), 200 mg quartz sand and 3 ml buffer A (HEPES, 0.1M pH 7.3; PEG-1500(Merck) 1.5%; Sucrose 10%; Dithioerythritol 1mM; Ascorbic Acid 100 mM; $CaCl_2$ 25mM) and again ground for 5 minutes in cold condition. The extract was then centrifuged at 20,000 g for 20 minutes and supernatant was stirred for 15 minutes with Dowex 1X2-200 pre-equilibrated with buffer A. Then again it was centrifuged to remove the resin and the supernatant was used for desalting over a Sephadex G -25 column pre-conditioned in buffer B (potassium Phosphate buffer, 0.1M, pH 7.0; Sucrose: 10%; Sodium ascorbate: 20mM). Finally, the crude enzyme was obtained by elution with 1 ml buffer B.

Assay of ANR activity

The activity of anthocyanidin reductase (ANR) was assayed according to method described by Punyasiri *et al*. (2004) with some modifications. The ANR reaction assay mixture included 50 µl of 20 mM NADPH, 100 µl saturated cyanidin chloride solution (1mg/ml H_2O), enzyme extract corresponding to 100 µg total protein, then volume made up by Tris-HCl buffer (pH 7.0) to final volume of 500 µl. The assay was incubated for 30 min at 45°C. The reaction products were extracted twice with 500 µl ethylacetate each time. Supernatants were then pooled and dried under vacuum and finally dissolved in 60 µl methyl alcohol for further analysis.

Paper chromatography of seed extract and reaction product

Total extractable phenol from seed tissue was extracted following the method described in Sinha et al. (2014). The solvent system used in paper chromatography separation was water: formic acid: ethyl acetate (1:1:18, v/v). The reference molecules used for identification of monomers were catechin and epicatechin solution as standard. The reaction products of ANR extracted in ethylacetate were analysed using paper chromatography. Different volumes of reaction products ranging from 20-100 µg enzyme extract were loaded for identification of the reaction product by paper chromatography using epicatechin as substrate. After the chromatography experiment chromatogram was sprayed with 0.1% (w/v) DMACA freshly prepared in 6M HCl: Ethanol (1:1).

Results and Discussion

In this study, we first standardized a protocol for high throughput staining of whole seed to assess the presence of tannin in the seed coat. The DMACA (p-dimethyl aminocinamaldehyde) staining clearly shows uniform presence of tannin in seed (Fig 1A) which was also confirmed by tannin deposition in seed coat histologically (Fig. 1B). Having confirmed the presence of CT in most important edible part of faba bean, it was now very important to determine its building block which could be either catechin or epicatechin (Xie et al. 2003). We then extracted total phenol from seed and separated them using paper chromatography in presence of catechin and epicatechin as reference. Our result results showed the presence of epicatechin in seed extract equivalent to epicatechin reference (Fig 2A). The equivalent spot of

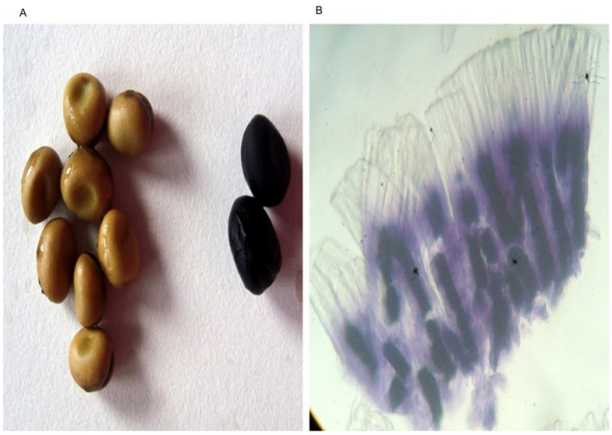

Fig 1. Staining of whole seed (A) and tissue section of seed coat (B) with DMACA

catechin was not observed. Antocyanidin reductase can catalyse the synthesis of cetechin or epicatechin using cyaniding as substrate (Xi *et al*. 2004). For further confirmation of epicatechin as building block we purified anthocyanidin reductase from leaf tissue of faba bean and

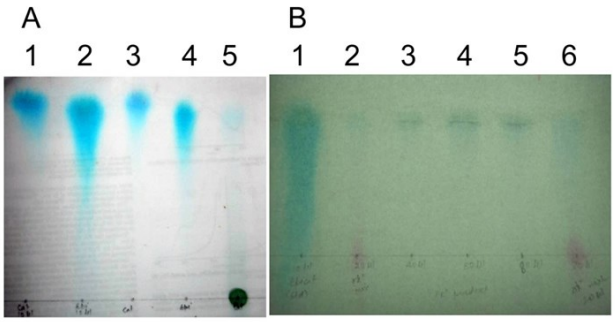

Fig 2. (A) Separation of monomers of seed extract: 1 and 3 are 5 and 10µl catechin standard respectively; 2 and 4 are 5 and 10 µl epicatechin standard respectively; 5 is 30 µl of seed extract. (B) 1 is 5 µl of epicatechin standard; 2-6 are reaction product consisting of 20-100 µg enzyme extract.

then we allowed *in-vitro* catalysis of ANR in presence of cyanididn as substrate. Then the reaction product extracted was resolved by paper chromatography in presence of epicatechin as reference. We found the presence of epicatechin in all reaction products containing enzyme extract of amount ranging from 60-100 µg (Fig. 2B). The presence of epicatechin spot demonstrated that epicatechin is the monomer of condensed tannin resulted from ANR catalysis present in seed coat.

The present study demonstrated that epicatechin is the building block substrate used for the biosynthesis of condensed tannin in faba bean at least in its seed coat which is responsible for astringent taste of seeds. Since we now know the building block of this polymer, the enzyme/gene responsible for biosynthesis of this monomer can be targeted to modulate the amount of condensed tannin in seed coat. There are various biotechnological tools now available which can either down-regulate the expression of gene responsible to epicatechin biosynthesis or completely knock-out the concerned gene in tissue specific and temporal basis of expression. However, the end product resulted from this approach should first be evaluated for its suitability in external environment.

Conclusions

Although presence of secondary metabolites in any plant parts has its own ecological importance but they often pose antinutritional attributes for human consumption. Condensed tannins slow down its commercialization. Present study identified the monomer of this large molecule which paves the way to improve faba bean either through breeding or genetic engineering approaches.

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