Identification of an isoflavone glycoside hydrolyzing β-glucosidase in the seeds of the desert legume Prosopis cineraria

V. Asati and P. K. Sharma*

Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani-333 031, Rajasthan, India.

ABSTRACT

Legumes, in general, are rich in glycosidic isoflavone conjugates, whereby specific β-glucosidases act to release the free aglycones that could serve in plant-microbe interactions and defense. Prosopis cineraria is an important legume tree, bearing medicinally useful and edible pods, generally growing in extreme arid/semi-arid zones of Rajasthan. However, specific phytochemical-cum-metabolic profiles are lacking for the same. Therefore, the present investigation undertook phytochemical and metabolic screening of the pods/seeds of P. cineraria for the presence of putative isoflavonoids viz. genistein and daidzein, their glycosides and β-glucosidase(s) capable of catalyzing the glycoside hydrolysis. Extraction and identification of these two aglycone forms of the above isoflavonoids were performed with solvent partition chromatography and Fluorescent/High Performance Thin Layer Chromatography, respectively. Furthermore, optimization of the isoflavone conjugate-specific β-glucosidase activity with respect to pH optima, temperature and time were carried out. The partially purified enzyme showed a temperature optima of 50°C and pH optima of 4.5. The enzyme also demonstrated activity towards natural substrates daidzin and genistin which are glycosides of isoflavonoids daidzein and genistein respectively. The methanolic extracts of the seeds of P. cineraria indicated the presence of related isoflavonoids which needs to be further validated.

Key words: Glycoside, β-Glucosidase, Isoflavonoid, Prosopis cineraria

INTRODUCTION

Prosopis cineraria (khejri) is an important legume tree growing in Rajasthan (India). The tree has immense historical significance as Bishnoi community near Jodhpur sacrificed themselves for its protection (Jain, 2011). Although medicinal properties viz. anticancer, antidiabetic, antioxidant etc have been attributed to its leaves, bark, flowers, roots as well as related agro-waste byproducts (Sharma et al., 2010; Beniwal and Jood, 2014), the pods (known as sangri) are the real edible parts for humans. Nutrition-wise, these contain significant amounts of vitamin C (Rathore, 2009). However, today many farmers tend to cut down the tree for firewood and short term monetary gains (Narayan and Kumar, 2015). It is high time the validation of the phytochemical and biochemical aspects of the tree is undertaken by the scientific community to further its categorization as a nutraceutical food source.

Although scattered reports do occur in literature vis-à-vis the natural product categories (flavonoids, terpenoids, alkaloids) for different parts of khejri (Purohit et al., 1979; Sharma et al., 2010), studies pertaining to specific active metabolites and their in planta biotransformation are few. An important medicinally valuable class of (phenolic) compounds present in legumes is the isoflavonoids. These can act as phytoestrogenic, anticancer, antibacterial, etc. (Chuankhayan et al., 2007). Some well-studied legumes in this regard are Glycine max (soybean), whose seeds possess therapeutically important (anticancerous and anti-oxidant) isoflavonoids like daidzein, genistein and glycitein (Zhu et al., 2005; Sinha et al., 2013), and Vicia faba to name a few (Sinha et al., 2013). However, since such phytoanticipins may be toxic to the synthesizing tissue itself, these are converted to 7-O-glycoside conjugates and stored in the embryo/seed coat/vacuoles (Lepiniec et al., 2006). When the plant needs the active metabolite, indigenous β-glucosidases release both the aglycone and the sugar.

Besides soybean, the isoflavone conjugate hydrolyzing β-glucosidases have been reported from chickpea (Hosel and Barz, 1975) and Thai rosewood (Srisomsap et al., 1996). In both cases, detailed characterization has been done and implications in plant defense aptly established. The present study was undertaken with a view to screen the pods/seeds of Prosopis cineraria for soybean-like isoflavonoids and corresponding enzymes involved in their metabolism. As per our knowledge, this is the first time an isoflavonoid conjugate hydrolyzing β-glucosidase is being reported from P. cineraria.

*Corresponding author’s e-mail: pankajsharma@pilani.bits-pilani.ac.in
MATERIALS AND METHODS

The pods of Prosopis cineraria were collected from local vendors in Jaipur, Rajasthan, India during April-May, 2015. Immediately after collection, they were shade-dried and stored at room temperature till further use. For the soybean extract, the seed powder was purchased from BITS Consumers and Cooperative Stores, Pilani.

For the purpose of extraction of isoflavonoids/related compounds from the pods, the seeds were finely powdered with a mixer-grinder, defatted thrice with hexane to remove pigments and lipids. Thereafter, three-times-extraction in 80% methanol was done with continuous vortexing in between. The methanol extracts were completely dried under vacuum and dissolved in absolute methanol for further analysis. Soybean extracts were prepared following the above mentioned procedure. The extracts from the above plants were used for the phytochemical analysis. The purpose of using soybean extract was to compare its isoflavonoid content with that of the P. cineraria.

For enzyme extraction, 30 gm of P. cineraria seed powder was homogenized with cold extraction buffer (phosphate buffer, pH 6). The crude extract obtained after centrifugation (10,000 rpm, 30 min, 4°C) was subjected to 30% ammonium sulfate precipitation. The supernatant obtained on centrifugation (10,000 rpm, 30 min, 4°C) was subjected to 70% ammonium sulfate precipitation and centrifuged. The resulting pellet was dissolved in 3 ml of extraction buffer and desalted using a column (25*1.8 cm) containing Sephadex G-25 (Sigma Aldrich). The resulting protein-rich eluate (as verified by OD 280 spectrophotometric readings; Jasco -V630 spectrophotometer) was used for enzyme assays.

For preliminary characterization studies of enzyme, the synthetic substrate PNPG (p-nitrophenyl-β-D-glucopyranoside) was used. The assay mixture contained 80 µl Citrate Phosphate Buffer (0.1 M, pH=4.5), 25 µl Substrate (10 mM Daidzin/Genistin) and 95 µl of enzyme preparation. Reaction mixture was incubated for 3 hrs at 50°C and was stopped using 200 µl methanol. Furthermore, the product formed was extracted thrice using chloroform. Resultant extracts were completely dried and were dissolved in absolute methanol. The product formation was checked using Thin Layer Chromatography (TLC). Negative control in the form of zero-time control was used where methanol was added before enzyme and then kept for incubation.

Simple TLC for detection of enzymatically derived product with natural isoflavonoid substrates was done on Silica gel F254 plates (Merck) using the solvent system Toluene: Ethyl Acetate: Acetone: Formic Acid: 20:4:2:1. For verifying the presence of isoflavonoids, using the same solvent system HPTLC was performed with methanolic extracts of P. cineraria seeds and soybean seeds by CAMAG TLC Scanner 3.

RESULTS AND DISCUSSION

The assay for isoflavonoid glycoside hydrolyzing β-glucosidase revealed that the seeds of P. cineraria (khejri) pods do contain enzyme activity (activity was absent in the pericarp), since spots corresponding to the putative aglycone products (daidzein and genistein, respectively) could be detected in the appropriate TLC lanes under UV 254 illumination (Figure 1a). Further kinetic studies need to be done to confirm if the activity is due to a generalized or an isoflavone conjugate-specific β-glucosidase. If the latter holds true, the present result could be of significance since till date, such specific β-glucosidases have been verified from only a few legumes such as soybean, chickpea and Thai rosewood (Hsieh and Graham, 2001; Hosel and Barz, 1975; Srisomsap et al., 1996). Nevertheless, the current finding points out to the fact that in khejri seeds, the glycoside-to-aglycone metabolism could be mediated by these enzymes, which might serve the seed during germination in hostile environments. The fact that the plant recruits such enzymes for isoflavonoid glycoside breakdown underlines important roles played by these metabolites for the plant. The released aglycone could protect the seed from both biotic and abiotic stresses while the released sugars could provide energy to the developing seedling. Preliminary biochemical characterization of the partially purified enzyme using the synthetic substrate PNPG showed the pH and temperature optima to be 4.5 and 50°C respectively, (Figures 1b and 1c). For comparison, the soybean root and chickpea seed enzymes have pH optima of 6.0 and 7.0, with temperature optima of 30°C and 45°C respectively (Hsieh and Graham, 2001; Hosel and Barz, 1975). Thus, enough variability is seen in biochemical parameters. Time-dependent assay showed that
there was a linear increase in enzymatic activity till 120 minutes. (Figure 1d) The coefficient of determination R² was observed as 0.9941 for the graph. Nonetheless, the commercial utility of such enzymes in enhancing isoflavonoid bioavailability in soy and similar ‘healthy flour’ extracts on account of releasing the more bioactive aglycone forms (Setchell et al., 2002; Chuankhayan et al., 2007) merits further purification and detailed characterization of the khejri enzyme.

Our next aim was to test whether isoflavonoids or similar metabolites are present in the seeds of khejri, an expectation also arising from the literature reports wherein
these compounds are more often than not exclusively present in the members of the Leguminosae family (Dewick, 1994). As seen from HPTLC results (Figure 2), comparing the $R_f$ values with those of standards and soybean metabolites suggests the presence of putative isoflavonoids in *P. cineraria* seeds. Daidzein and Genistein are the isoflavonoids which were chosen as the standards in the above experiments. There has been enough research where these compounds have been reported in *Glycine max* (soybean) in significant quantity. It has been already established that the health promoting properties of *Glycine max* can be attributed to the presence of such important compounds (Li et al., 2005). Considering the dietary benefits bestowed by isoflavonoids, this result of the preliminary phytochemical analysis could be of immense significance for non-soybean eating human populations (Wang et al., 2013). It has been highlighted that *P. cineraria* holds an inevitable importance in the flora of the arid region of Rajasthan (Singh, 2009). Therefore, shedding light on the phytochemical and biochemical properties of this legume will help in their protection amidst changing traditional values.

CONCLUSION

The present study looked at the possible presence of health-benefitting soybean-like isoflavonoids and corresponding glycoside hydrolases in the seeds of khejri plant, scattered over many dry areas of Rajasthan, India. A $\alpha$-glucosidase capable of hydrolyzing the isoflavonoid glycosides daidzin and genistin was partially characterized. HPTLC analysis indicated the presence of corresponding aglycones, viz. daidzein and genistein. However, a note of caution is warranted here. Since concluding the presence of isoflavonoids in *P. cineraria* seeds based solely on the HPTLC $R_f$ values might not be 100% accurate, further advanced studies need to be performed to verify the same. This might include large scale purification using preparative column chromatography and structural studies employing Mass spectrometry, Infrared spectroscopy and Nuclear Magnetic Resonance techniques. Also further purification and complete biochemical characterization of the purified $\alpha$-glucosidase should be another extension of the above study.

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