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Isolation and Determination of Benzo(a)pyrene Glucuronide and Sulfate Conjugates in Soybean Leaves

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Many authors (Lao et al. 1973, Blumer et al. 1977, Crosby et al. 1981, Dunn 1976) have reported the presence of polycyclic aromatic hydrocarbons (PAH) in the environment. Of these PAH, benzo(a)pyrene (BaP) is particularly carcinogenic. BaP is metabolized in mammalian systems by the mixed function oxidase system of liver microsomes. This system catalyzes the oxidation of BaP via epoxide intermediate to phenol, diol and quinone metabolites. One of these, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BaP is thought to act as the ultimate carcinogen by binding covalently to cellular DNA (Slaga et al. 1977). It is also known that Cunninghamella elegans oxidized BaP to its phenol, diol and quinone metabolites. In addition, the alcohols were detected as glucuronide and sulfate conjugates (Cerniglia and Gibson 1979). These metabolites are remarkably similar to those observed in higher organisms. On the other hand, some investigators have demonstrated that plants take up BaP and anthracene from soil or culture medium containing these compounds (Shiraishi et al. 1982, Edwards et al. 1982). This paper reports the finding that soybeans grown in BaP polluted soil take it up and metabolize to its phenol, diol and the glucuronide and sulfate conjugates of the alcohols.

MATERIALS AND METHODS

9,10-Dihydroxy-9,10-dihydro-BaP (BaP-9,10-diol), 7,8-dihydroxy-7,8-dihydro-BaP (BaP-7,8-diol) and 3-hydroxy-BaP (BaP-3-monool) were kindly provided by Dr. Longfellow, National Cancer Institute, The carcinogen Standard Reference Repository, Bethesda, Maryland. 9-Hydroxy-BaP (BaP-9-monool) was prepared by reported method (Sims 1967). β- Glucuronidase, type H-1 (EC 3.2.1.31) and saccharic acid-1,4-lactone were from Sigma Chem. Co., St., Mo. Other chemicals were obtained from Wako Chem. Co., Tokyo.

Soybean seeds (Glycine max) were grown in a greenhouse (Sakamoto et al. 1984). The leaves (after 76 days growth) were homogenized with two volumes (w/v) of chloroform-methanol (2:1) and filtered.

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The residue was extracted again 3 times. All extracts were combined and upper methanol-water layer was removed. The lower chloroform-layer was washed with a half volume of methanol-water (9:1). The washed upper phase was combined with the methanol-water layer and concentrated in vacuo on a rotary evaporator.

Pre-purification of the concentrate (2.1 g, one fourth of the concentrate) was done by column chromatography on alumina without activation (200 mesh, 50 g, glass column, 20 x 400 mm). Eluting solvent was 400 ml of methanol. Further purification of the eluate (80 mg, conjugates) was carried out by thin-layer chromatography (TLC) on Wako gel B5 using n-propanol-pyridine-water (7:4:2) as developing solvent (detection; UV lamp, yield; 73 mg). For purification of BaP monool and diol(s) produced by enzyme reaction of the conjugates was done by TLC developed with benzene-ethanol (9:1). Separation of the alcohols is described below.

Glucuronide conjugates were detected by sprying TLC plates with 50 % sulfuric acid and heating; the unidentified conjugate was detected by sprying with ninhydrin reagent in the usual manner. Conjugates containing glutathione or amino acid may react positively.

Two stoppered conical centrifuge tubes containing 12.2 mg of the conjugates, 5.0 ml of 0.2 M sodium acetate buffer ($_{p}$H 5.5) and 1,000 units of β-glucuronidase (one of the tubes contained no enzyme) were incubated at 37°C in the dark for 24 hr in a water bath incubator operated at 100 strokes/min. The tubes were extracted with 15 ml of ethyl acetate. The upper layer was dried on a rotary evaporator in vacuo and used for analysis of BaP alcohols (Cerniglia and Gibson 1979).

The tubes containing 10.3 mg of the conjugates (described above), 5 ml of 0.1 M phosphate buffer ($_{p}$H 7.5), 12.5 u moles of D-saccharic acid-1,4-lactone and 10 units of arylsulfatase (EC 3.1.6.1, Sigma) were incubated at 37°C for 24 hr. The reaction mixture was extracted with 15 ml of ethyl acetate and analyzed.

Hitachi Model 638 High Pressure Liquid Chromatograph fitted with Hitachi gel 3053 column (2.5 x 500 mm, Permaphase ODS type) was used without gradient elution. The solvent composition was acetonitrile-water (60:40) and the effluent was monitored at 254 nm. Flow rate was 0.5 ml/min at 200-300 Kg/cm^2 and the oven temperature was 30°C.

Mass spectra (direct probe injection method) were recorded at an ionization voltage of 50 eV (Hitachi Model RMU 6MGC).

RESULTS AND DISCUSSION

The conjugates which were fluorescent showed R_f values between 0.57 and 0.67 on a TLC plate developed with n-propanol- pyridine-water (7:4:2). The color reaction of the upper part of the spots with 50 % H_2SO_4 was violet and of the lower part of the spots with ninhydrin reagent was purple. It was reported that the

Table 1. Characteristics of BaP alcohols prepared from soybean leaves

Compound	TLC [1] R_f	HPLC [2] retention time (min)	m/z [2]	Precent [3]
Standard BaP alcohols				
BaP-9,10-diol	0.25	6.47	286 (9), 268 (100), 239 (53).	——
BaP-7,8-diol	0.32	9.09	286 (10),268 (100), 239 (40).	——
BaP-9-monool	0.62	16.80	268 (100),239 (51).	——
BaP-3-monool	0.63	18.09	268 (100),239 (51).	
Soybean alcohols				
A	0.26	6.44	286 (10), 268 (100), 239 (38).	84.8
B	0.63	16.61	268 (100), 239 (34)	15.2

1) developing solvent : chloroform-ethanol (9:1).
2) Condition : see text.
3) In the case of BaP glucuronine.

aqueous fraction of BaP incubated with rat liver homogenate contained a glutathione conjugate that formed a fluorescent band and gave a purple color with ninhydrin reagent (Waterfall and Sims 1972). The R_f values of the BaP alcohols from soybean leaves with chloroform-methanol (9:1) are shown in Table 1. Two bands, BaP-9,10-diol (A) and BaP monool (B) were detected as compared to standard compounds.

High pressure liquid chromatography (HPLC) elution data and other characteristics of the BaP alcohols are also shown in Table 1. Each compound was separated on TLC plate and purified by HPLC. The ultraviolet spectrum of compound A was identical with that of standard BaP-9,10-diol (Cerniglia and Gibson 1979, Waterfall and Sims 1972) This result was supported by mass spectral data which showed an intensity molecular ion at m/z 286. In addition, compound B identified as BaP-9-monool (m/z 268) by the same method.

The yield of mixed BaP alcohols after β-glucuronidase treatment was 3.1 mg (41 % as BaP-diol glucuronide) and with arylsulfatase was 0.8 mg (10 % as BaP-monool sulfate). The liberated BaP alcohol composition from both enzyme reaction was the same; ratio of A : B, 84.8 : 15.2

These results demonstrated that a higher plant can oxidize BaP to its alcohols that are qualitatively similar to those produced by mammalian microsomes (Slaga et al. 1977) and eukaryotic microorganism (Cerniglia and Gibson 1979). The formation of BaP-9,10-diol and BaP-9-monool as major plant metabolites suggest that a mixed function oxidase catalyzed the preformation of BaP-9,10-epoxide (Waterfall and Sims 1972). Cunninghamella elegans produced BaP-9,10-diol, BaP-7,8-diol, BaP-1,6- and 3,6-quinones, BaP-9- and 3-monools (Cernigria and Gibson 1979). It has been reported that soybean leaves grown in BaP polluted soil contained BaP-1,6-, 3,6- and 6,12-quinones (Sakamoto et al. 1984). It is interesting to note that soybean leaves produce BaP alcohols and quinones that correspond to metabolites of higher organisms, glucuronide and sulfate conjugates as detoxification products. Their role in fungi has yet to be determined but they could conceivably be a similar.

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