PRODUCTS OF THE PLANT METABOLISM OF PCB

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Abstract

Plant cells were screened for their ability to transform polychlorinated biphenyls (PCB). As model plant tissue cultures cultivated *in vitro* were used. Four plant species (horseradish, black nightshade, alfalfa and tobacco) were chosen for detailed study of the metabolism and intermediates formed. Hydroxychlorobiphenyls were identified as the main products of transformation of PCB by plant cells, low amounts of diphenylethers and chlorobenzoic acids were detected. Hydroxychlorobiphenyls are probably mostly formed by cytochrome P450, nevertheless due to similar reaction and substrate specifity also peroxidases can be involved. Further studies have shown that isolatéd peroxidases from black nightshade and tobacco are able to efficiently transform monochloro- and dichlorobiphenyls. As the products of this transformation benzoic and hydroxybenzoic acids, compounds like phenylacylchlorides were identified. Also more chlorinated and dechlorinated products were obtained.

Introduction

Some microorganisms (bacteria and fungi) were found to be able to metabolise polychlorinated biphenyls (PCBs)(1). Recently also plants were shown to be able to transform these xenobiotics to non-phytotoxic compounds (2-5). In contrast to bacteria the knowledge about plant metabolism involved in these processes is limited. In our studies plant tissue cultures of different plant species cultivated *in vitro* were used to evaluate the ability of plant cells to metabolise PCBs. As a model PCB system a recalcitrant mixture of PCB congeners with different degree of chlorination – Delor 103 and individual mono – and dichlorobiphenyls were used (6, 7). Differentiated and transformed plant tissue cultures metabolized PCBs with higher efficiency than amorphous and nondifferentiated ones of the same species. For metabolic studies, 4 plant tissue cultures of different species – hairy root culture *Solanum nigrum* (black nightshade), embryogenic culture *Armoracia rusticana* (horseradish) and callus cultures *Nicotiana tabacum* (tobacco) and *Medicago sativa* (alfalfa) were chosen and transformation of individual mono- and dichlorobiphenyls congeners was followed. Mostly hydroxychlorobiphenyls were detected as products of PCB conversion in plant cells, probably formed in reactions catalysed by cytochrome P450. Further experiments showed also ability of plant peroxidases to transform PCBs. Halogenation, dehalogenation but also degradation of biphenyl structure was documented.

Materials and Methods

Plant Cell Cultures

In vitro cultures of four plant species (alfalfa, black nightshade, tobacco and horseradish) exhibiting different morphology (non-differentiated amorphous callus strains and hairy root clones transformed by Ti or Ri plasmids of *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively) were from the collection of the Department of Natural Products, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic.

Cultivation conditions

Plant cell cultures were incubated aseptically in Murashige and Skoog's (1962) nutrient medium for 14 days on rotary shaker (100 rpm) in the dark at 24ºC. According to the experiments, usually 5 g cell wet

weight was used as inoculum, and cultivation carried out in 100 ml liquid media in 250 ml Erlenmayer flasks.

PCBs
A standard commercial mixture of PCBs, Delor 103 (D) was used as methanol solution. This mixture contains about 60 individual congeners of PCBs substituted by 1-5 chlorine atoms per biphenyl molecule. The initial concentration of Delor 103 was 25 ppm. For the enzymatic reactions and identification of products formed individual congeners of mono- and dichlorobiphenyls (Dr. Ehrensdorfer GmbH, Germany) were used in initial concentration of 5 – 10 µM (equivalent to 1,11 - 1,88 ppm). After incubation the residual amount of PCBs was analysed by gas chromatography (Burkhard et al., 1997), the structures of metabolites were identified after silylation (9) by GC-MS and comparison with the structure of standards.

Analysis of residual content of PCBs
After the cultivation, the cells were killed by boiling for 20 min., sonicated, homogenised and the whole content of flask was extracted with hexane for 2 hours (7, 8). Decrease of PCB concentration was measured by gas chromatography with ECD, expressed as percentage of PCB removed comparing to control flasks (11). Flasks with heat-killed cells (90ºC, 25 minutes) were used as controls and treated the same way as those containing living cells.

Extraction and purification of plant peroxidases, estimation of POX activity
To estimate the changes in peroxidase activity, additional flasks with the same content of inoculum were incubated parallely with the samples for estimation of PCB transformation. The flasks contained 2.5 - 5 mg of the mixture Delor 103 (+D), individual PCB congeners in concentration of 0.3 mg/100 ml in each flask, or no Delor 103 (-D). Cell extracts were prepared by homogenization of 1 g frozen cell fresh weight by pestle and mortar with 1 ml of 0.1 M phosphate buffer (pH 6.5) and centrifugation for 15 minutes at 5000 rpm. The total peroxidase activity in the cell extract and in the medium was measured by the spectrophotometric method using guaiacol and hydrogen peroxide at 470 nm described by Chromá et al. (6). The isoenzyme pattern of peroxidases in the cell extract and in the medium was analysed by native electrophoresis in polyacrylamide gel after visualisation of peroxidase isoenzymes with guaiacol and hydrogen peroxide (6,11).

Concentration and partial purification of intracellular peroxidase
Proteins from black nightshade and tobacco suspended in extraction buffer were precipitated and partially purified by salting out (40-80% saturation of ammonium sulphate). After precipitation protein suspension was centrifuged (10 min., 10 000 rpm) and desalted by gel chromatography on commercial columns PD-10 (Pharmacia) containing Sephadex G-25 as a resin.

<u>In vitro</u> reactions of peroxidases with individual congeners of PCBs
Concentrated peroxidase preparations were tested for their transformation potential towards individual PCB congeners. Twelve different individual congeners of monochlorobiphenyls and dichlorobiphenyls in concentrations of 5-10 µmol/l (see Table 1) were used as substrates for POX from black nightshade, four of them for POX isolated from tobacco cells (see Table 3). Reactions were started by the addition of hydrogen peroxide. After 22 hours of the reaction at 25^{o}C residual peroxidase activity was inactivated by 1 ml of sulphuric acid (10 %) and remaining PCBs were extracted by hexane (7) and analysed by GC with ECD (7).

Results and Discussion
The metabolism of polychlorinated biphenyls (PCB) and the ability to transform these compounds by plant tissue cultures of four plant species was studied in laboratory and also field scale. Level of transformation has been followed by estimations of the decrease of content of PCB congeners, with different degree of chlorination, contained in commercial mixture Delor 103 and by estimation of the decrease of particular congeners of mono- and dichlorobiphenyls. The decrease of PCB congeners after biotransformation was detected by gas chromatography with EC detector. Twenty-one cultures of

different plant species and different morphological differentiation were examined for the ability to transform polychlorinated biphenyls. Differentiated plant cultures and/or plant cultures transformed by wild-type plasmid from *Agrobacterium* have shown higher capability to metabolise PCB congeners. These cultures also better tolerated toxic effect of PCB, which resulted in higher cell growth. Plant cultures *Solanum nigrum* SNC 90 (black nightshade, hairy root type), *Medicago sativa* Alf (alfalfa, amorphous callus), *Nicotiana tabacum* WSC 38 (tobacco, amorphous callus) and *Armoracia rusticana* K54 (a shoot-forming horse-radish culture) have been chosen as model systems to monitor biotransformation of monochlorobiphenyl congeners. Tobacco has shown the highest degree of monochlorobiphenyls (2-, 3- and 4-chlorobiphenyl) conversion. In the case of hairy root clone SNC 9O of *Solanum nigrum* the biotransformation of dichlorobiphenyl congeners (PCB 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15) have been followed. 84 % of PCB congener 4 (2,2´-dichlorobiphenyl) has been found after biotransformation, congeners PCB 10 (2,6-dichlorobiphenyl) and PCB 6 (2,3´-dichlorobiphenyl) have been metabolised to the residual amount of 87 % and 89 %, respectively. Very low transformation has been found in the case of the congeners PCB 13, PCB 14 and PCB 15. Cells of above mentioned cultures of different plant species (*Armoracia rusticana-horseradish*, *Medicago sativa-alfalfa*, *Solanum nigrum-black nightshade* and *Nicotiana tabacum-tobacco*) cultivated *in vitro* have been chosen for monitoring of monochlorobiphenyls metabolism (PCB 1, 2, 3) and analysis of intermediates. Hydroxychlorobiphenyls have been generated as essential products of the metabolism of said monochlorobiphenyls. Several of their products were identified by comparison with mass spectra of the standards. Above all it was 5-hydroxy-2-chlorobiphenyl, which is formed after the cultivation of *Armoracia rusticana* and *Solanum nigrum* with PCB 1, 4-hydroxy-3-chlorobiphenyl and 6-hydroxy-3-chlorobiphenyl, both identified after the cultivation of *Armoracia rusticana* and *Solanum nigrum* with PCB 2, and 4´-hydroxy-4-chlorobiphenyl, formed after the cultivation of all plant cultures with PCB 3. In the case of plant culture *Medicago sativa* no other hydroxychlorobiphenyls have been identified, except 4´-hydroxy-4-chlorobiphenyl. Analysis and comparison of the toxicity of monochlorobiphenyls and hydroxychlorobiphenyls as initial products of PCB metabolism in plants have shown, that intermediates - hydroxychloroderivatives are more toxic, probably due to higher solubility than original chlorinated compounds. Concerning enzyme participation on PCB metabolism it is known that mostly cytochrome P450 is involved in transformation of xenobiotics. Recently in literature it was described that also peroxidases can participate in metabolism of various xenobiotics including PCB (9). Three cultures (hairy root of black nightshade, callus of tobacco and callus of alfalfa) synthesized both intracellular and extracellular peroxidases showing the same or similar isoenzyme patterns when incubated without PCBs. Peroxidases of tobacco and black nightshade changed in the presence of PCB their total POX activities and some changes in isoenzyme patterns were also visible after native electrophoresis. Total peroxidase activity of alfalfa exhibited the highest values without PCB presence, but its activity and isoenzyme pattern was not influenced by the presence of PCBs and this plant was generally less active in PCB transformation (6). Thus in further experiments we tested only intracellular peroxidases from tobacco and black nightshade cells, of which crude extracts were partly purified and concentrated by ammonium sulphate precipitation (40-80 % saturation) prior to reactions with PCBs. Both peroxidase activities per ml of preparations exhibited similar values – 0.89 nkat/ml for black nightshade and 0.87 nkat/ml for tobacco, respectively. Twelve different individual congeners of PCBs were used as substrates for POX from black nightshade, four of them for POX isolated from tobacco cells (Table 1).

Decrease of PCBs concentrations after the reactions with POXs is shown in Table 1. Parallely reaction mixtures with the same PCB congeners treated the same way as described above were extracted with toluene and after silylation (9) of the reaction products analysed by GC-MS.

From Table 1 it can be seen that the efficiency of degradation of chosen congeners was similar with both peroxidases. Activities of POXs decreased during whole reaction time (22 hours) with all chosen congeners to 0.009-0.013 nkat/ml, i.e. 910 times. Efficiency of degradation of structurally different PCB is, with the highest probability, connected with their chemical structure, physico-chemical properties and also toxicity (see Table 1). Comparing three monochlorobiphenyls the most efficient transformation of PCB was estimated in reaction mixture containing PCB 3. This congener exhibited also the lowest toxicity measured by the growth of plant hairy roots (10), compare to resulting transformation and toxicity of monochlorobiphenyls, the values also corresponded except for PCB 5. This congener proved inhibition coefficient higher than 30 (10) (i.e. it is relatively toxic), but on the other hand it was efficiently metabolised by 63%.

Small quantities of numerous products formed during reactions of POX isolated from black nightshade with PCBs were detected by GC-MS.

Table 3: Degradation of PCB by intracellular plant peroxidases isolated from the culture of black nightshade and tobacco

IUPAC No.	PCB	toxicity expressed as inhibition coefficient (%)*	degradation of PCB by POX from black nightshade (%)	degradation of PCB by POX from tobacco (%)
PCB 1	2-Cl	18,0	35±5	-
PCB 2	3-Cl	15,0	35±4	-
PCB 3	4-Cl	8,0	100±8	79±10
PCB 4	2,2´-Cl	24,4	56±7	60±8
PCB 5	2,3 -Cl	34.8	63±8	-
PCB 7	2,4 -Cl	19,3	59±7	47±6
PCB 8	2,4´-Cl	37,1	16±2	-
PCB 9	2,5 -Cl	20,1	66±7	-
PCB 10	2,6 -Cl	25,2	14±2	-
PCB 11	3,3´-Cl	21,9	28±4	-
PCB 14	3,5 -Cl	51,8	16±2	12±2
PCB 15	4,4´- Cl	50,6	8±1	-

* inhibition coefficient was calculated as the growth of the roots without toxicant minus growth in presence of toxicant divided by the value of growth without presence of toxicant

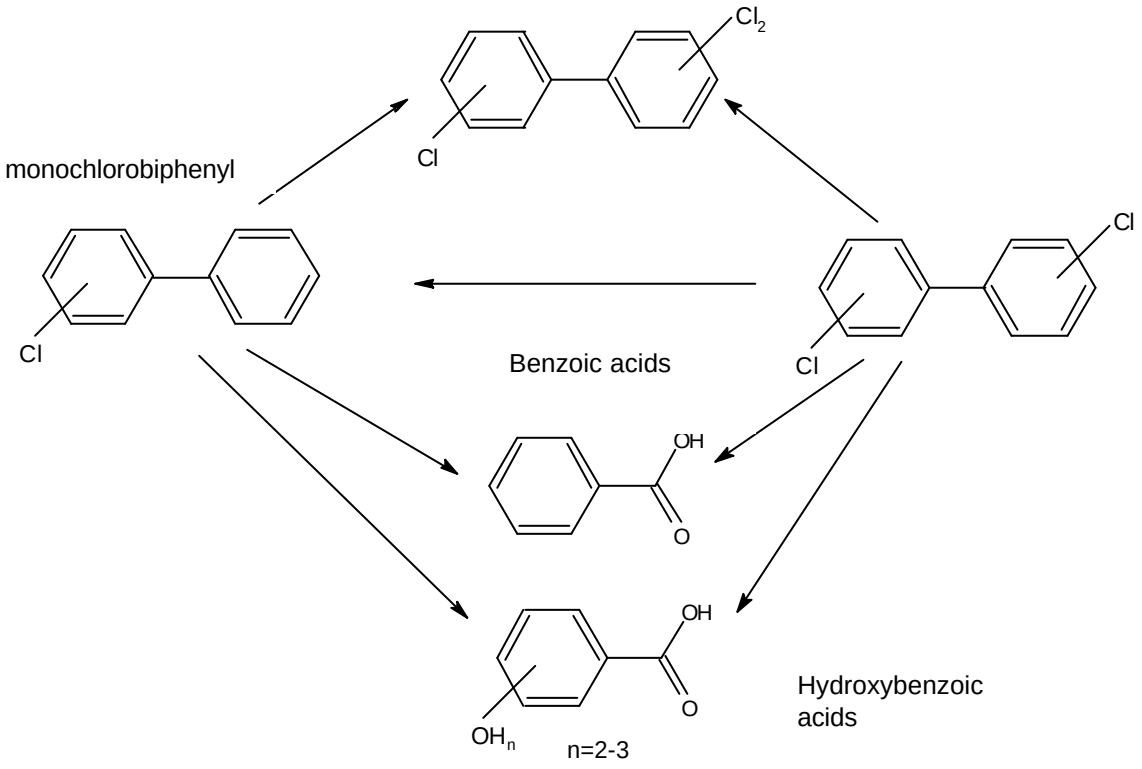

Figure 1: Reaction scheme of plant peroxidases – transformation of PCB

Unfortunately reactions with tobacco POX did not give any detectable products. The concentration of products was probably under detection limit. In reaction mixtures containing POX from black nightshade, dechlorination was observed as the initial step. As metabolites less chlorinated biphenyls than the original ones or non-substituted biphenyls were detected. Koller et al. (9) showed, after reaction of commercial HRP with PCBs, beside formation of other metabolites, presence of very low concentrations of chlorohydroxybiphenyls.

In our study suprisingly no hydroxylated chlorobiphenyls were found. This phenomenon can be explained by further fast reactions following the first dechlorination step without any accumulation of intermediates (9). Regarding oxidative degradation, the cleavage of the ring system and subsequent reactions gave benzoic acid and hydroxybenzoic acids as the products. As a result of POX radical mechanism higher chlorinated and less chlorinated isomers of biphenyls were formed. Traces of phenylacylchlorides were also found in reaction mixtures (6).

Conclusions

From above mentioned results can be concluded that plants transform PCBs using different oxidative enzymes, cytochrome P450 and probably also peroxidases. While cytochromes are not produced as extracellular proteins, peroxidases can transform compounds inside and also outside the cells. As mentioned elsewhere (2, 5), bioavailability of organics in soils appears to be primary restriction for effective phytoremediation, thus extracellular synthesis of transforming enzymes appears to be an additional efficient strategy contributing to enhancement of biodegradation processes by plants.

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References

(1) Abramowicz, M., Aerobic and anaerobic PCB biodegradation in the environment. Environmental Health Perspectives **103,** 97-99, (1990).

(2) Alkorta, I., Garbisu, C., Phytoremediation of organic contaminants in soils. Bioresource Technology **79**, 273-276, (2001).

(3) Bock, C., Kolb, M., Bokern, M., Harms ,H., Mackova, M., Chroma, L., Macek, T., Hughes, J., Just, C., and Schnoor, J., Advances in phytoremediation: Phytotransformation. PCB – approaches to possible removal from the environment. (Reible D. and Demnmerova K., eds.), NATO ASI Series, Kluwer Academic Publishers, Dordrecht, pp. 115-140, (2002).

(4) Macek, T., Macková, M., Káš, J, Exploitation of plants for the removal of organics in environmental remediation. Biotechnology Advances **18**, 23-35, (2000).

(5) Schnoor, J. L., Licht, L. A., McCutcheon, S. C., Wolfe, N.L. and Carreira, L. H., Phytoremediation of organic and nutrient contaminants. Environmental Science and Technology **29**, 318-323, (1995).

(6) Chroma, L., Mackova, M., Kucerova, P., in der Wiesche, C., Burkhard, J., Macek, T., Enzymes in plant metabolism of PCBs nad PAHs. Acta Biotechnologica **22**, 34-41, (2002).

(7) Mackova, M., Macek, T., Ocenaskova, J., Burkhard, J., Demnerova, K., Pazlarova, J., Biodegradation of polychlorinated biphenyls by plant cells. International Biodeterioration and Biodegradation **39**, 317-325, (1997).

(8) Ryslava, E., Krejcík, Z., Macek, T., Novakova, H. and Mackova, M., Study of PCB biodegradation in real contaminated soil. Fresenius Environmental Bulletin **12**, 296-301, (2003).

(9) Koeller, G., Moeder, M., Czihal, K., Peroxidative degradation of selected PCB: a mechanistic study. Chemosphere **41**, 1827-1834, (2000).

(10) Lovecka, P., Melenova, I., Kucerova, P., Nováková, H., Macková, M., Ruml, T., Demnerová, K., Evaluation of ecotoxicity of PCB and their bacterial and plant degradation products using different biological systems. In: Book of Abstracts, The Second PCB Workshop, (Holoubek, Holoubkova eds.), pp. 32, (2002).

(11) Kucerova, P., Mackova, M., Chroma, L., Burkhard, J., Triska, J., Demnerova, K., Macek, T., Metabolism of polychlorinated biphenyls by *Solanum nigrum* hairy root clone SNC-9O and analysis of transformation products. Plant and Soil **225**, 109-115, (2000).