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Enzymatic hydrolysis of structurally diverse phthalic acid esters by porcine and bovine pancreatic cholesterol esterases

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ABSTRACT

A weak hydrolyzing activity against bis (2-ethylhexyl) phthalate (DEHP) was discovered in a commercial crude lipase (EC 3.1.1.3) preparation from porcine pancreas. DEHP was hydrolyzed to mono (2-ethylhexyl) phthalate (MEHP) not by a pancreatic lipase but by a cholesterol esterase (CEase, EC 3.1.1.13), a trace contaminant in the crude lipase preparation. Enzymatic hydrolysis of phthalic acid esters (PAEs), suspected to be endocrine-disrupting chemicals, was investigated using CEases from two species of mammals and a microorganism. Eight structurally diverse PAEs, namely diethyl phthalate (DEP), di-*n*-propyl phthalate (DPrP), di-*n*-butyl phthalate (DBP), di-*n*-pentyl phthalate (DPeP), di-*n*-hexyl phthalate (DHP), DEHP, *n*-butyl benzyl phthalate (BBP), and dicyclohexyl phthalate (DCHP), were hydrolyzed to their corresponding monoesters by both porcine and bovine pancreatic CEases, while a microbial CEase from *Pseudomonas* sp. had no hydrolyzing activity against these PAEs. The hydrolysis experiments with bovine pancreatic CEase (50 U) indicated complete hydrolysis of every PAE (5 μmole) except for BBP and DCHP within 15 min; BBP and DCHP were hydrolyzed within 30 min and 6 h, respectively. The rates of PAE hydrolysis could be affected by the bulkiness of alkyl side chains in the PAEs. This study provides important evidence that mammalian pancreatic CEases, such as those from porcine and bovine sources, are potential enzymes for nonspecific degradation of structurally diverse PAEs.

1. Introduction

Phthalic acid esters (PAEs) are among the most important industrial chemicals widely used in various industrial products, such as plastics, coatings, cosmetics, as well as plasticizers to make polyvinylchloride (PVC) pliable. Because of their wide commercial applications, large scale production, and leachability from products, PAEs have become ubiquitous environmental pollutants, exerting a noticeable impact on the ecology (Bauer et al., 1998).

Humans are exposed to PAEs from food contaminated during processing, and packaging or from indoor air (Matsumoto et al., 2008). Several in vitro studies have recently revealed that PAEs such as di-*n*-butyl phthalate (DBP) and *n*-butyl benzyl phthalate (BBP) are capable of binding to estrogen-receptor α (ER_α), inducing ER_α-mediated gene expression and enhancing the proliferation of MCF-7 human breast cancer cells (Jobling et al., 1995; Harris et al., 1997; Zacharewski et al., 1998; Takeuchi et al., 2005). These results have changed the fundamental perception of PAEs as potential health hazards endocrine-disrupting chemicals (EDCs) that interfere with the reproductive systems of humans and wildlife by mimicking estrogens. Several regulatory bodies, such as the United States Environmental Protection Agency (EPA, 1991), have designated PAEs as top priority pollutants that require risk assessments and have mandated their reduction and control in the environment (Xu et al., 2005). The Environment Agency of Japan has compiled a priority list of compounds, named SPEED '98 (JEA, 1998), and listed the following eight PAEs as suspected EDCs in 1998: diethyl phthalate (DEP), di-*n*-propyl phthalate (DPrP), DBP, di-*n*-pentyl phthalate (DPeP), di-*n*-hexyl phthalate (DHP), bis (2-ethylhexyl) phthalate (DEHP), BBP, and dicyclohexyl phthalate (DCHP).

PAEs are susceptible to hydrolysis in abiotic environments. They can undergo sequential hydrolyses of their ester linkages by first forming their corresponding monoesters and then phthalic acid (PA). However, the chemical hydrolysis rates of PAEs either are very slow or are negligible, especially under neutral and acidic conditions (Staples et al., 1997). Therefore, metabolic breakdown of PAEs by microorganisms is believed to be a major means for degrading these widespread pollutants in the environment (Chatterjee and Dutta, 2003).

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Many microorganisms with PAE metabolizing abilities have been isolated from diverse environments, such as soil, natural water, and wastewater (Staples et al., 1997). Some microorganisms selectively hydrolyze only one ester bond to produce a monoalkyl phthalate and an alcohol, while others are capable of complete mineralization of either monoalkyl or dialkyl phthalates (Chatterjee and Dutta, 2003). Some major enzymes involved in microbial metabolism of PAEs, such as phthalate oxygenase, phthalate dioxygenase, phthalate dehydrogenase, and phthalate decarboxylase have been identified (Kurane et al., 1980, 1984; Nomura et al., 1992).

Previous research suggests that metabolic pathways for microbial metabolism of PAEs initiate with a deesterification reaction to form their corresponding monoesters and alcohols under both aerobic and anaerobic conditions in some cases (Staples et al., 1997; Niazi et al., 2001). To date, there have been a few reports that studied first-step hydrolysis of PAEs to their corresponding monoesters and alcohols using esterases from microorganisms. Kurane et al. investigated the effect of esterases from *Norcardia erythropolis* in hydrolyzing DEHP to PA (Kurane et al., 1984). Niazi et al. reported the initial degradation of dimethyl phthalate (DMP) by esterases from *Bacillus* species (Niazi et al., 2001). However, only several reports have investigated PAE hydrolysis by mammalian enzymes, such as nonspecific lipase from rat pancreas (Albro and Thomas, 1973; Albro and Latimer, 1974; Albro et al., 1976), esterases from rat small intestine (White et al., 1980), and carboxylesterases from rat and human (Mentlein and Butte, 1989).

In this study, enzymatic hydrolysis of DEHP, the most commonly used PVC plasticizer, was examined using commercial lipase preparations from microorganisms, plants, and animals. From these results, a CEase from porcine pancreas, a trace contaminant in the porcine pancreatic lipase preparation, was revealed to have strong DEHP-hydrolyzing activity. Based on this observation, enzymatic hydrolyses of the eight PAEs listed in SPEED '98 (including DEHP) was performed with mammalian CEases from porcine and bovine pancreases to confirm that the potential of these CEases in inducing initial enzymatic degradation of the structurally diverse PAEs to their corresponding monoesters.

2. Materials and methods

2.1. Chemicals

PAEs (DEP, DPrP, DBP, DPeP, DHP, DEHP, BBP, and DCHP) of phthalate standard grades, monoethyl phthalate (MEP), and PA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mono-*n*-butyl phthalate (MBuP), monobenzyl phthalate (MBeP), and mono (2-ethylhexyl) phthalate (MEHP) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used in this study were of reagent grade.

2.2. Estrogenic activity assay

Competitive binding assays for human estrogen-receptor α (ERα) used an Estrogen-R (α) Competitor Screening kit (Wako). These assays were performed as previously described (Satoh et al., 2001). In brief, solutions of fluorescent-labeled 17β-estradiol (E2) and chemicals were added to the wells of a plate coated with human ERα and were then allowed to compete for 2 h at room temperature. The plates were washed with a wash solution, after which the assay solution was added to each well. Fluorescence intensity was detected using a microplate fluoresence photometer (excitation at 485 nm, emission at 535 nm).

2.3. Enzymes

Lipase preparations from *Rhizopus arrhizus*, *Mucor javanicus*, *Mucor miehei*, wheat germ, and porcine pancreas (Type II: 147 U mg^{-1} protein, Type VI-S: 35,500 U mg^{-1} protein) were obtained from Sigma Chemical Co. (St. Louis, Mo.). CEase preparations from porcine (31 U mg^{-1} protein) and bovine (24.2 U mg^{-1} protein) pancreases, as well as *Pseudomonas* sp. (21 U mg^{-1}) were obtained from Sigma, Oriental Yeast Co., Ltd. (Osaka, Japan) and Wako, respectively. All enzymes were used as received without any further purification.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the procedure of Laemmli (1970) with 10% polyacrylamide gel (TEFCO, Tokyo, Japan). Samples (5 μg) were boiled in the presence of 2-mercaptoethanol for 5 min before their application onto the gel. Protein bands were visualized by Coomassie Brilliant Blue R-250 (Sigma) staining. Molecular weight was determined using a molecular weight marker kit (Bio-Rad Laboratories, Inc., Hercules, CA).

2.5. Enzymatic reaction

A reaction mixture containing enzyme, PAE, organic solvent, and a surface active agent in 50 mM phosphate buffer (pH 7.0) was incubated at 37 °C. A PAE was solubilized in an organic solvent, which was either methanol or DMSO. Polyoxyethylene (9) lauryl ether (BL-9EX) was also used for completely solubilizing a PAE. After incubating the reaction mixtures for a maximum of 24 h, the reaction was stopped by adding 10 vol.% of 1 N HCl to the mixture, and the reaction products were extracted using the same volume of ethyl acetate.

2.6. Reversed-phase high-performance liquid chromatography (HPLC)

For reversed-phase HPLC analysis, the extracts were dehydrated with anhydrous $MgSO_4$, filtered, evaporated, and dissolved in ethanol. HPLC used a cosmosil $5C_{18}$-MS-II AR column (4.6 mm I.D. × 150 mm, Nacalai Tesque, Kyoto, Japan) packed with a silica gel (particle size: 5 μm; pore size: 120 Å) bonded with octadecyl group (monomeric) and endcapped. The mobile phase was methanol/water/phosphoric acid (95/5/0.1 v/v) at a flow rate of 0.5 mL min^{-1} at 30 °C. The eluent was monitored by ultraviolet absorbance (254 nm). The residual PAE (%) was determined by calculating the portion of PAE before and after the reaction.

2.7. Thin layer chromatography (TLC)

The extracted reaction products were separated on a silica gel 60 F_{254} (Merck, Darmstadt, Germany) reversed-phase TLC plate, and developed in *n*-hexane/ethyl acetate/glacial acetic acid (50/50/0.5 v/v). After the plates had been developed, they were examined under UV light, and the locations of the developed compounds were photographed. For quantitative analysis, the photographs were scanned using a flatbed scanner, and band intensities were analyzed using ImageJ 1.41. The residual PAE (%) was determined by calculating the portion of PAE before and after the reaction.