Characterization of Human Salivary Esterase in Enzymatic Hydrolysis of Phthalate Esters

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Enzymatic hydrolysis of phthalate esters in human saliva was investigated to characterize salivary esterase in the formation of monoesters from their diesters. The monoesters formed were analyzed by GC/MS after incubation of phthalate diesters in the saliva. Hydrolytic activity in the supernatant obtained by centrifugation of the saliva at 1350 × *g* was equivalent to that in whole saliva, and the activity was inhibited by the addition of denaturing protein. The hydrolytic activity was dependent on the protein concentration in the supernatant. The optimum temperature and pH of the hydrolysis was 50°C and 8.2, respectively. In addition, the 80% acetone powder of the supernatant showed high substrate specificity for straight-chain alkyl group of phthalate diesters, especially the butyl group, whereas almost no specificity was seen for the 2-ethylhexyl and benzyl groups. These results indicate that not the oral flora but salivary esterase, such as lingual lipase, is involved in phthalate monoester formation from the diesters in human saliva, and do not act on the hydrolysis of monoester.

Key words —— human saliva, phthalate diester, hydrolysis, phthalate monoester, salivary esterase, acetone powder

INTRODUCTION

Phthalate diesters are widely used as plasticizers to impart softness and flexibility to normally rigid plastics such as polyvinyl chloride (PVC). Medical devices and toys are often made of PVC and contain the predominant plasticizers di-iso-nonyl phthalate, di-2-ethylhexyl phthalate (DEHP), and/or di-*n*-butyl phthalate (DBP).[1)]

Some phthalate diesters induce testicular toxicity in the male reproductive tract. Jobling *et al.* showed that DBP binds to the estrogen receptor, displaces its natural ligand, and then acts as an estrogen antagonist.[2)] In addition, mono-*n*-butyl phthalate (MBuP) adversely affects development of the reproductive system in male rats.[3)] DEHP is a peroxisome proliferator that induces tumors in the rodent liver, and this effect is probably not exerted by DEHP itself but by one or more of its metabolites.[4)]

Phthalate diesters are metabolized to monoesters by hydrolases in many tissues, such as the pancreas, intestine, and blood.[5–7)] Generally orally ingested dialkyl phthalates are hydrolyzed by esterases in the wall of the small intestine and pancreatic lipases and not by gut flora. Absorption occurs almost entirely as the corresponding monoesters.[5)] Human saliva is a matrix of inorganic compounds, saliva proteins such as lingual lipase and α-amylase, oral flora, and cell debris.[8)] However, to the best of our knowledge there are no reports concerning enzymatic hydrolysis of phthalate diesters in human saliva, except for our previous paper.[9)] The present work was therefore designed to characterize human salivary esterase in enzymatic hydrolysis of phthalate esters. These findings provide the information that will be required in considering the human health effects of phthalate esters.

MATERIALS AND METHODS

Chemicals and Glassware —— Monoethyl phthalate (MEP), diethyl phthalate (DEP), and DEHP were purchased from Wako Pure Chemical Industries. (Osaka, Japan) and MBuP, mono-*n*-hexyl phthalate (MHxP), mono-2-ethylhexyl phthalate (MEHP), monobenzyl phthalate (MBeP), and di-*n*-hexyl ph-

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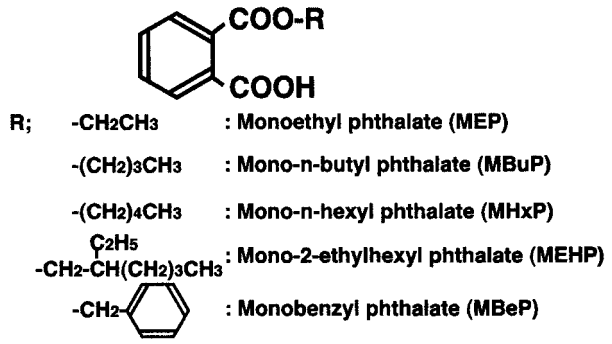

Fig. 1. Chemical Structures of Phthalate Monoesters

thalate (DHxP) were from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). DBP and butyl-benzyl phthalate (BBP) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). The purity of MEP, MBuP, MHxP, and MEHP was over 90%, that of MBeP was 98%, and that of DEP, DBP, DHxP, DEHP, and BBP was over 99.0%. Trimethylsilyldiazomethane (TMSD) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The structure of phthalate monoesters is shown in Fig. 1.

Tris–HCl buffer solution (pH 7.2) was prepared as follows: Trishydroxymethyl-aminomethane (25 ml of 24.3 mg/ml [0.2 M], Wako Pure Chemical Industries) was mixed with 45 ml of 0.1 M hydrochloric acid, adjusted to pH 7.2, and then adjusted to 100 ml with distilled water. Sodium dodecyl sulfate (SDS, 1 g; Wako Pure Chemical Industries) was dissolved in a final volume of 100 ml of the Tris–HCl buffer solution (pH 7.2). Monoesters were solid-phase extracted using Oasis hydrophilic lipophilic balance (HLB) cartridges (3 cc) from Waters Co. (Milford, MA, U.S.A.). The cartridges were washed immediately before use with 5 ml of ethyl acetate, followed by 5 ml of methanol and 10 ml of 0.5% acetic acid. All other solvents and reagents were of analytical grade, and confirmed as being phthalate diester free. Glassware was heated at temperatures above 230°C for at least 5 hr before use.

Human Saliva and Enzyme Preparations —— Fifteen adult volunteers (10 men, 5 women) gave informed consent for participation in all procedures associated with the study. The volunteers mouthed and chewed a polypropylene disk (2.5 × 3.0 cm) without phthalate diesters for 15 min, then all saliva produced in the oral cavity was collected into centrifugation tubes between 09:00 and 10:00. The volume and pH of the collected saliva ranged from 7 to 21 ml and from 7.3 to 7.9, respectively. The saliva was centrifuged at 3000 rpm (1500 × *g*) for 20 min below –5°C. The supernatant was filtered through a 0.45 μm membrane (Advantec MFS, Inc., U.S.A.), and the precipitate was suspended in 10 ml of Tris–HCl buffer solution (pH 7.2).

Acetone powder (30%, 60%, and 80%; crude enzyme) was prepared from the supernatant of saliva as follows. Acetone (30%, 60%, and 80% v/v) was slowly added to the supernatant at –5°C, and then the mixture was centrifuged at 3000 rpm (1500 × *g*) for 30 min at –5°C. The supernatant was removed and the precipitate was dried under a nitrogen gas stream. The precipitates (acetone powder) were stored at –20°C. Porcine pancreatic lipase (EC 3.1.1.3), porcine liver carboxyl esterase (EC 3.1.1.1), human saliva α-amylase (EC 3.2.1.1), and human milk lysozyme (EC 3.2.1.17) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Instruments —— GC/MS analysis was performed by the method described in the previous paper.[10]

HPLC operating conditions required to detect phthalic acid and phthalate monoesters were as follows: apparatus, LC-10A (Shimadzu, Kyoto, Japan); column, Inertsil C8-3 (0.46 mm ϕ × 250 mm); column oven temperature, 40°C; mobile phase, 0.1% acetic acid including 5.0 mmol/l tetra-*n*-butylammonium phosphate solution/acetonitrile (50 : 50); detector, UV (254 nm); and injection volume, 10 μl.

Determination of Enzyme Activity in Saliva and Enzyme Source —— Human saliva or enzyme source (1.0 ml) was added to the same volume of the Tris–HCl buffer solution (pH 7.2) and then incubated with 50 nmol of phthalate di- or monoesters in 10 μl of DMSO for 30 min. The incubation temperature was 37°C and pH of the saliva or enzyme source was 7.2 in this experiments, in order to mimic the hydrolysis in human oral cavity. The reaction was stopped by adding 0.1 M hydrochloric acid (0.1 ml) and acetonitrile (1 ml). Saliva was mixed with 8 ml of 0.1% acetic acid, loaded onto Oasis HLB cartridges, and rinsed with 5 ml of distilled water/methanol (9 : 1). Organic compounds remaining in the column were eluted with 10 ml of methanol/ethyl acetate (1 : 9) and evaporated to dryness under nitrogen gas. Ethyl acetate (0.5 ml) and 100 μl methanol were added to the residue, which was then derivatized with TMSD 30 μl at ambient temperature for 30 min.[10] The solvent was evaporated to dryness under nitrogen gas, and the residues were dissolved in *n*-hexane 1 ml. The methylated monoesters were quantified by GC/MS. The phthalic acid composition of acidified saliva was determined

Table 1. Amounts of Mono-*n*-Butyl Phthalate Formed by the Hydrolysis of Di-*n*-Butyl Phthalate in the Human Saliva Incubated at 37°C for 30 min

Human saliva	Additions	DBP[d)]	DBP + SDS[e)]
	Products	Amount of MBuP (nmol)	
Whole saliva[a)]		22.4±9.8	< 0.1
Supernatant[b)]		19.0±9.1	< 0.1
Precipitate[c)]		4.8±3.3	< 0.1

a) Human saliva obtained from volunteers. *b*) Supernatant of the human saliva centrifugated at 3000 rpm, −5°C for 20 min, and filtered through a 0.45-μm filter. *c*) Precipitate of human saliva centrifugated at 3000 rpm, −5°C for 20 min, and suspended with Tris–HCl buffer solution (pH 7.2). *d*) Human saliva 1 ml was added to Tris–HCl buffer (pH 7.2) 1 ml and DBP 50 nmol and shaken at 37°C for 30 min. *e*) Human saliva 1 ml was added to SDS (1% SDS in Tris–HCl buffer solution) solution 1 ml and DBP 50 nmol and shaken at 37°C for 30 min.

by HPLC immediately after incubation. Protein was determined by the color reaction with pyrocatechol violet-molybdenum (IV) complex method[11] using the Micro TP-Test (Wako Pure Chemical Industries).

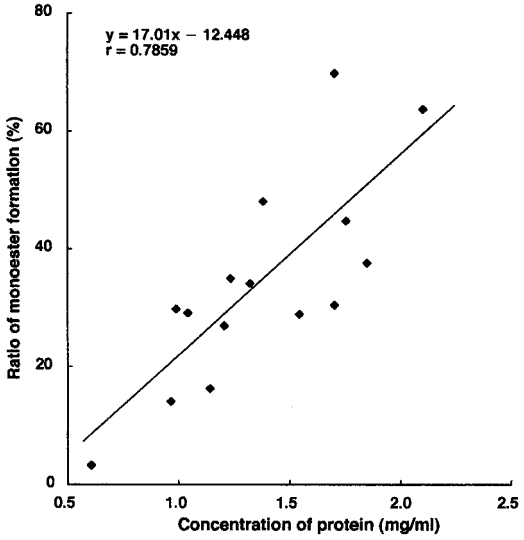

Fig. 2. Correlation of Protein Concentration in Saliva from Each Volunteer with the Ratio of Mono-*n*-Butyl Phthalate (MBuP) Formation from Di-*n*-Butyl Phthalate (DBP)

Incubation conditions: Saliva sample (1 ml) was shaken with Tris–HCl buffer solution (pH 7.2) 1 ml and DBP 50 nmol at 37°C for 30 min. Human saliva samples produced by chewing a polyethylene disk were centrifuged at 3000 rpm at –5°C for 20 min, and then filtered through 0.45-μm membranes. Filtrates were used as saliva samples.

RESULTS AND DISCUSSION

Effect of Hydrolase in Human Saliva on the Formation of Phthalate Monoesters from Diesters

The composition of human saliva varies due to the proportions of 1) inorganic compounds and salivary proteins secreted from the salivary gland, and 2) oral bacteria, and cell and food debris.[8] The saliva was centrifuged to separate the two sets of components to confirm in which phthalate diesters were hydrolyzed. Table 1 shows the effects of saliva on DBP hydrolysis. The ratios of MBuP formation in the supernatant and precipitate of saliva were 84.8% and 21.4%, respectively. Hydrolysis activity was found in the soluble fraction of saliva, but the activity disappeared upon the addition of 1% SDS–Tris–HCl buffer solution to the soluble fraction.

Salivary supernatant (100% v/v) was diluted with Tris–HCl buffer solution to ratios of 75%, 50%, and 25% (v/v). The ratios of MBuP formation in the 100%, 75%, 50%, and 25% (v/v) supernatant were 78%, 58%, 39%, and 20%, respectively. The concentration of supernatant was proportional to the formation ratio. Figure 2 shows the effect of the protein concentration on the MBuP formation in individual supernatants from 15 volunteers. The amounts of MBuP formation and the concentration of protein in the supernatant were significantly correlated (correlation coefficient = 0.79). It has been known that human saliva contains α-amylase, lingual lipase, and lysozyme, which are all hydrolases.[8] The hydrolytic activity forming phthalate diesters depended strongly on the amount of hydrolase in the saliva.

Enzymatic Properties of Salivary Hydrolase for Phthalate Esters

Figure 3 shows the effect of temperature and pH on DBP hydrolysis in the supernatant of saliva. The optimum temperature and pH of DBP hydrolysis was 50°C and 8.2, respectively. These results were in agreement with the optimum temperature at which porcine pancreatic lipase forms tributyrin and triolein in the presence of taurocholic acid.[12] It has also been reported that the optimal pH values for lipase to form triglycerides in the human pancreas, duodenum, and milk are 8.0, 6–9, and 8–9, respectively.[13]

The hydrolysis of phthalate di-esters by human salivary hydrolase is shown in Table 2. The hydrolytic activity in 30% acetone powder was almost zero. The substrate specificity of the 80% acetone powder for dialkyl esters was similar to that of the supernatant. MBuP and MBeP were formed from BBP in the supernatant and the acetone powder. Little hydrolysis activity was observed in the benzyl group

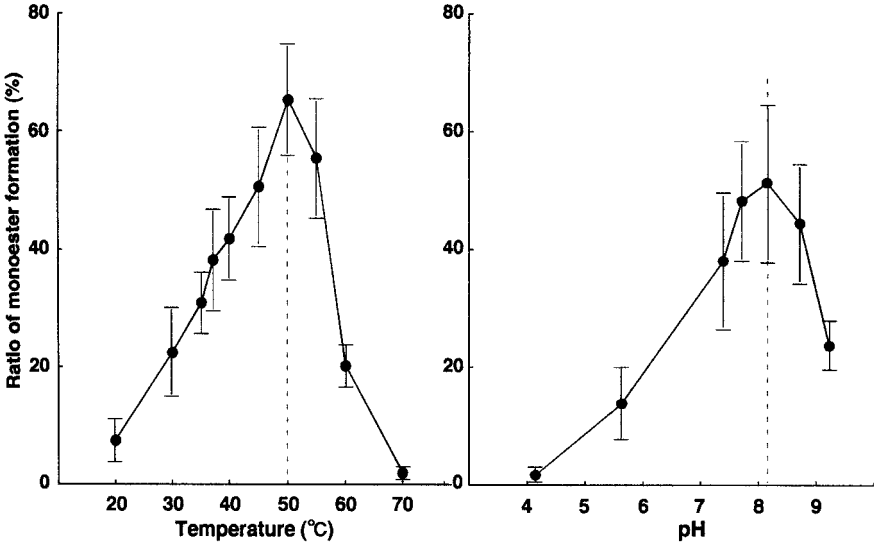

Fig. 3. Effects of Temperature and pH of Saliva on the Ratio of Mono-*n*-Butyl Phthalate (MBuP) Formation from Di-*n*-Butyl Phthalate (DBP)

Incubation conditions: Saliva sample (1 ml) was shaken with Tris–HCl buffer solution 1 ml and DBP 50 nmol for 30 min. Human saliva samples produced by chewing a polyethylene disk were centrifugated at 3000 rpm at –5°C for 20 min, and then filtered through 0.45-μm membranes. Each point indicates the mean value (n = 5).

Table 2. Formation of Phthalate Monoesters from Diesters by Hydrolase in Human Saliva

Enzyme preparation[a)]	Substrate	DEP	DBP	DHxP	DEHP	BBP		MBuP
	Product	MEP	MBuP	MHxP	MEHP	MBuP	MBeP	PA
		Hydrolysis activity (nmol/mg protein)						
Saliva sample		5.9	12.4	8.6	0.5	0.4	2	< 0.1
30% Acetone powder		< 0.1	0.4	0.2	< 0.1	< 0.1	< 0.1	< 0.1
60% Acetone powder		1.2	9.4	3.6	0.3	0.2	3	< 0.1
80% Acetone powder		3.9	10.7	6.2	0.4	0.3	2.9	< 0.1
Purified hydrolase								
Lipase from porcine pancreatine		3.6	5.8	6.4	0.5	0.4	2.8	< 0.1
Esterase from porcine liver		17.3	14.5	9.2	3.8	0.6	3.5	0.1
α-Amylase from human saliva		< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Lysozyme from human milk		< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

a) Details of preparation: see text. Enzyme preparation 1 ml was added to Tris–HCl buffer (pH 7.2) 1 ml and substrate 50 nmol and shaken for 30 min. Each value indicates the mean (n = 5).

of BBP, whereas that of the butyl group was marked. The hydrolase in human saliva had high substrate specificity for straight chains of phthalate diesters, especially the butyl group, but little for the 2-ethylhexyl and benzyl groups. Phthalic acid was not formed by hydrolysis of phthalate monoesters in either the supernatant or acetone powder. The solubility of phthalate esters in the saliva appears to be closely associated with their enzymatic hydrolysis.

Hydrolysis of phthalate esters was determined using commercially purified hydrolases that are present in the human saliva (Table 2). While activities were recognized in pancreatic lipase and liver carboxylesterase, they were not completely recognized in salivary α-amylase and milk lysozyme. The similarity of the hydrolysis activity between the acetone powder of human saliva and the purified lipase was higher than that of the purified carboxylesterase. Phthalate diesters have been monohydrolyzed by pancreatic lipase and intestinal esterase from a rodent and a primate species.[5,7)] Albro and Thomas concluded that orally ingested phthalate diesters were absorbed from the human gut primarily as the corresponding monoester derivatives.[14)]

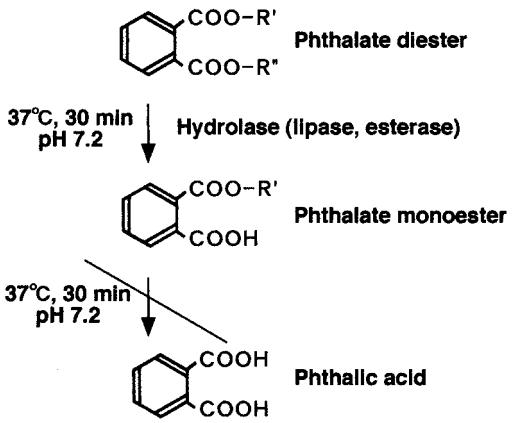

Fig. 4. Hydrolysis of Phthalate Esters by Hydrolase in Human Saliva

Monoesters were not hydrolyzed to phthalic acid by salivary hydrolase.

We found that some phthalate diesters in the oral cavity were hydrolyzed to monoesters by salivary esterase. The monoesters might therefore be incorporated *in vivo* with the saliva.

Crude lipase can be precipitated by 35% to 65% acetone from biological sample at temperatures of less than 4°C.[15] Lipase from cattle milk hydrolyzes hydrophobic tributyrin, but is inactive against hydrophilic monoesters.[16] In addition, lingual lipase in human saliva hydrolyzes triglyceride, and it seems to play an important role in the digestion of milk fat by newborns.[17] On the basis of these findings, esterase or lingual lipase appears to be closely involved in phthalate monoester formation from hydrophobic diesters in human saliva, but this enzyme barely hydrolyzes hydrophilic monoesters (Fig. 4).

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