Sulfation of Parabens and Tyrosylpeptides by Bacterial Arylsulfate Sulfotransferases

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Arylsulfate sulfotransferase purified from *Eubacterium* A-44 has higher specific activity than the enzymes from *Klebsiella* K-36 and *Haemophilus* K-12. Propylparaben and butylparaben were good substrates among several parabens. The antibacterial activity of parabens was reduced by the sulfation of the phenolic hydroxy group. Tyrosine-containing peptides, kyotorphin, enkephalin and cholecystokinin non-sulfate, were effective as acceptor substrates by A-44, K-36 and K-12 sulfotransferases.

Keywords arylsulfate sulfotransferase; paraben; tyrosylpeptide; intestinal bacteria

Modification of antibiotics and antiseptics with microorganisms, for example, by acetylation, phosphorylation and adenylation, is considered to be one of the significant inactivation processes of antimicrobial agents.¹⁾ In addition, sulfoconjugation is considered to be a major mechanism for the detoxification of endogenous and exogenous compounds bearing phenolic functional groups.^{2,3)} Relating to the sulfoconjugation reaction, liver, kidney, lung, erythrocyte and intestinal epithelial cells are generally regarded as the major organs and cells of detoxification.⁴⁻⁶⁾ Recently, we suggested that arylsulfate sulfotransferases (ASST) of intestinal bacteria are related to the metabolism of some phenolic compounds.^{7,8)} However, the substrate specificity of ASST of intestinal bacteria were quite different from the enzymes of mammalian organs. Although the donor substrate of the tissue enzyme was 3'-phosphoadenosine 5'-phosphosulfate (PAPS), donors of the bacterial enzymes were phenylsulfate esters.⁹⁾ Acceptor substrate specificities of the mammalian sulfotransferases have been studied in detail, but those of bacterial ASST have not been well elucidated.

The present study was undertaken in an attempt to sulfate some parabens and tyrosylpeptides with bacterial ASST.

MATERIALS AND METHODS

Materials Parabens, *p*-nitrophenylsulfate (PNS), bovine serum albumin and tyrosylpeptides were purchased from Sigma Chem. (U.S.A.). Paraben sulfate esters were synthesized by the ASST of *Eubacterium* A-44 and purified by silica gel column chromatography (solvents: butanol/H₂O/acetic acid (25/10/4)).

The Preparation of Feces Samples from Humans and Animals Fresh feces were collected from 3 healthy men and pooled. Male rats (Wistar, body weight 180–200 g) were maintained on pellet food (Samyang, Korea) and tap water *ad lib.* as one group of three to five. The feces of the rats were collected and pooled. Each feces was immediately suspended in 10 ml of 25 mM phosphate buffer, pH 7.0. The suspended samples were centrifuged at 500 × *g* for 5 min at 4°C, and the supernatants were

used for the enzyme solution.

Assay of ASST Activity The assay mixture (total volume of 0.63 ml) contained 0.29 ml of 1 mM phenol (occasionally other acceptors), 0.03 ml of 50 mM PNS, 0.21 ml of 0.1 M Tris-HCl buffer, pH 8.0, and 0.1 ml of the enzyme. After incubation of the assay mixture at 37°C for 15 min, the absorbance at 405 nm measured.

One unit of enzyme activity was defined as the amount required to catalyze the formation of 1 μmol of product per min under standard assay conditions. Specific activity was defined in terms of units per mg protein.

Protein Determination Protein was determined by the method of Lowry *et al.* by using bovine serum albumin as the standard.¹⁰⁾

Purification of Three Kinds of ASST The bacterium *Eubacterium* A-44, isolated from human feces, was precultured in a medium (50 ml General Anaerobic Medium (GAM) broth), inoculated in 2 l of GAM broth containing PNS as an inducer and cultured at 37°C for 18 h under an anaerobic condition. The cultured medium was centrifuged at 7000 rpm for 20 min at 4°C. The harvested cells were washed with saline, suspended with 0.1 M acetate buffer, pH 6.0, and disrupted by a sonicator (Heat Systems Ultrasonics, U.S.A.). Further steps of purification were carried out according to the previous method.¹¹⁾ The purified enzyme was 25.6 units/min/mg protein.

The bacterium *Klebsiella* K-36, isolated from rat intestinal bacteria, was inoculated in 10 l Luria-Bertani's broth. The cultured medium was centrifuged at 7000 rpm for 20 min at 4°C. The other steps of purification were carried out according to the previous method.¹²⁾ The purified enzyme was 5.8 units/min/mg protein.

The bacterium *Haemophilus* K-12, isolated from mouse intestinal bacteria, was inoculated in 10 l broth. The cultured medium was centrifuged at 7000 rpm for 20 min at 4°C. The harvested cells were washed with saline, suspended with 0.1 M phosphate buffer (pH 7.0), disrupted by a sonicator (Heat Systems Ultrasonics, U.S.A.) and then centrifuged at 13000 rpm for 30 min. The supernatant was applied to DEAE-cellulose column chromatography, poly(amino ethyl)-silica gel column chromatography and

Sephacryl S-300 fine column chromatography. The purified enzyme was 2.1 units/min/mg protein.

Assay of Antimicrobial Activity The antifungal activity was assayed on Sabouraud agar plates containing ethylparaben, propylparaben, ethylparaben *O*-sulfate or propylparaben-*O*-sulfate at various concentrations. *Candida albicans* or *Aspergillus niger* were inoculated in the agar plates and cultured at 30 °C for 2–3 d.

Antibacterial activity was assayed on Muller-Hinton agar plates containing parabens or paraben sulfate esters. *Staphylococcus aureus* was cultured at 37 °C for 22 h. Minimum inhibitory concentrations (MIC) of the test compounds were measured.

RESULTS

ASST of Intestinal Microflora When intestinal microflora of human and rat were cultured in GAM broth with various pHs (5, 6, 7, 8, 9 and 10), the best production of ASST was observed at pH 7–8 of the media. In the cases of *Eubacterium* A-44 and *Klebsiella* K-36 isolated from intestinal microflora, the pH of the medium for the best production of the enzyme was 7. However, the optimal pH for ASST activities of the feces of human and rats was 8–9, and those for *Eubacterium* A-44 isolated from human feces and *Klebsiella* K-36 isolated from rat feces were 8 and 10, respectively.

Sulfation of Parabens and Tyrosylpeptides by Bacterial ASST In order to investigate the sulfoconjugation of several parabens and tyrosylpeptides, we assayed the acceptor substrate specificity of these phenolic compounds using PNS as a donor substrate by bacterial ASST (Table I). The enzyme purified from *Eubacterium* A-44 has the highest specific activity in comparison with those purified from *Klebsiella* K-36 and *Haemophilus* K-12. Alkylparabens, in general, were good substrates as acceptors of A-44 ASST. Among the parabens, propylparaben and butylparaben were the best substrates. However, alkylparabens were not good substrates as acceptors of K-36 and K-12 ASST.

2,4,6-Trihydroxybenzoic acid was a better substrate than *p*-hydroxybenzoic acid, but 3,5-dihydroxybenzoic acid was worse than *p*-hydroxybenzoic acid. Tyrosine-containing peptides, such as kyotorphin, Leu-enkephalin and cholecystokinin non-sulfate, were effectively sulfated by A-44, K-36 and K-12 ASST, although the substrate specificity was different. In the case of A-44 ASST, similar to the previous results,¹³⁾ enkephalin was the best substrate, followed by kyotorphin and cholecystokinin non-sulfate. In the cases of K-36 and K-12 ASST, however, the best substrate was kyotorphin.

Optimal pH When 1-naphthol and phenol were used as acceptors, the optimal pH for A-44, K-36 and K-12 ASST were 8, 10 and 10, respectively. However, when tyrosylpeptides, enkephalin and cholecystokinin non-sulfate were used as acceptors, those for A-44, K-36 and K-12 ASST were all pH 8.

Antimicrobial Activity of Paraben Sulfate The MICs of ethylparaben and propylparaben for *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* were 250 µg/ml. However, those of ethylparaben *O*-sulfate and

TABLE I. Acceptor Substrate Specificity of Paraben Derivatives

Compound	ASST activity (%) ^{a)}		
	A-44	K-36	K-12
Phenol	100	100	100
Methylparaben	149	14	4.0
Ethylparaben	223	17	10.2
Propylparaben	255	20	12.5
Butylparaben	254	19	11.8
<i>p</i> -Hydroxybenzoic acid	10.3	13	10.4
3,5-Dihydroxybenzoic acid	3.7	5.6	6.2
2,4,6-Trihydroxybenzoic acid	39.1	14.6	12.5

Donor substrate: PNS. a) Specific activities of ASST from A-44, K-36 and K-12 were 25.6, 5.8 and 2.1 µmol/min/mg protein, respectively, under standard assay conditions which were taken as 100% in each column.

TABLE II. Sulfation of Tyrosylpeptides by Bacterial ASST

Compound	ASST activity (%) ^{a)}		
	A-44	K-36	K-12
Phenol	100	100	100
Tyrosine	0.13	14.3	5.2
Kyotorphin	4.0	28.6	11.2
Leu-enkephalin	5.8	4.3	10.5
Cholecystokinin nonsulfate	1.2	13.2	4.5

a) The ASST specific activities were the same as shown in Table I.

propylparaben *O*-sulfate were >500 µg/ml. The sulfated parabens were stable during incubation at 37 °C for 3 d in the control assay medium. The antibacterial activity of parabens was reduced by sulfation of the phenolic hydroxy group.

DISCUSSION

These bacterial sulfotransferases differ from the known mammalian enzymes in donor substrate specificity. That is, the bacterial ASST catalyzes the transfer reaction of a sulfate group from phenolic sulfate esters, but not from PAPS, which is a donor substrate of tissue sulfotransferase, to other phenols. Among three kinds of bacterial ASST, *Eubacterium* A-44 produced, in general, higher ASST activity on the sulfation of alkyl paraben than the other two enzymes, as shown in Table I. However, K-36 and K-12 strains produced higher ASST activity on the sulfation of tyrosine and tyrosine-containing peptides than A-44. Parabens was sulfated well by ASST of intestinal bacteria and antimicrobial activities of sulfated parabens were reduced. The result differed from that of amoxicillin.¹¹⁾ That is, the sulfation of parabens, we think, is the reaction of detoxification, occasionally the resistant mechanism of intestinal bacteria against paraben. It is considered that the sulfate groups are alternately transferred from phenolic sulfate esters to other phenols in the intestine by bacterial ASST, and that intestinal flora may play important roles in the metabolism and detoxification of phenolic compounds.

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