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Rat intestinal microbiota digest desulfosinigrin to form allyl cyanide and 1-cyano-2,3-epithiopropane

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ABSTRACT

The ability of rat intestinal microbiota to digest sinigrin and desulfosinigrin was studied using high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS). When sinigrin was incubated with rat intestinal microbiota, up to 64% of the initial amount of sinigrin was degraded during 12 h, yielding allyl isothiocyanate (AITC) as the major product along with a minor amount of allyl cyanide (ACN) and a trace amount of 1-cyano-2,3-epithiopropane (CETP), although the amount of ACN exceeded that of AITC after 12 h of incubation. In contrast, when desulfosinigrin was incubated with rat intestinal microbiota for 6 h, desulfosinigrin was digested up to 69% to form ACN and CETP as the major products instead of AITC. Whether the epithiospecifier protein (ESP) is involved in CETP formation in the rat intestinal microbiota-mediated degradation of desulfosinigrin remains to be established. However, only a trace amount of desulfosinigrin was detected during incubation of sinigrin with intestinal microbiota, although the intestinal microbiota had distinct sulfatase activity when *p*-nitrocatechol sulfate was used as the substrate. This observation suggested that the sulfatase of the rat intestinal microbiota has a very poor specificity toward sinigrin, which may be one reason for the formation of trace amounts of CETP during the intestinal microbiota-mediated digestion of sinigrin.

1. Introduction

Glucosinolates, one of the classes of phytochemicals occurring predominantly in the family Brassicaceae, undergo thioglucosidase (EC 3:2:1:147)-mediated hydrolysis to yield isothiocyanates and nitriles, including cyano-epithioalkanes and oxazolidine-2-thions, depending on their chemical structures and the enzymatic hydrolysis conditions, such as the medium pH and the presence of ferrous ion or epithiospecifier protein (ESP) in the medium (Bones & Rossiter, 2006; Halkier & Gershenzon, 2006). Among the organic products, a great deal of attention has been focused on isothiocyanates because of their notable activities in the inhibition of phase I procarcinogen activation enzymes (Conaway, Jiao, & Chung, 1996; Guo et al., 1992; Mahéo et al., 1997; Thornalley, 2002) and induction of phase II carcinogen detoxification enzymes (Fahey, Zalcmann, & Talalay, 2001; Holst & Williamson, 2004; Nakamura et al., 2000; Talalay, 1992; Zhang & Talalay, 1998). These activities of isothiocyanates have been implicated as chemopreventive in various cancers. In fact, the intake of cruciferous vegetables has been associated with a reduced risk of cancer of the breast, lung, forestomach, and esophagus (Bianchini & Vainio, 2004; Verhoeven, Verhagen, Goldbohm, van den Brandt, & van Poppel, 1997; Zhang & Talalay, 1994). On the other hand, nitriles, particularly epithionitriles, administered once daily to rats at high doses for 1 to 3 days have been reported to show toxic effects such as hepatocellular megalocytosis, renal necrosis, and ulceration in the forestomach (Wallig, Groud, Fettman, & Willhite, 1988). In other experiments, allyl cyanide (ACN) has been shown to induce behavioral abnormalities in mice at considerably higher doses than that associated with the daily vegetable consumption (Tanii, Takayasu, Higashi, Leng, & Saijoh, 2004). They also reported that allyl cyanide acts as an inducer of antioxidant and detoxification enzymes (Tanii, Higashi, Nishimura, Higuchi, & Saijoh, 2008). In addition, in the last two years, researchers have shown that 1-cyano-2,3-epithiopropane (CETP) and allyl isothiocyanate (AITC) behave as cancer chemopreventive phytochemicals (Kelleher et al., 2009; Zhang, 2010). When chopped or crushed raw cruciferous vegetables are ingested, the released isothiocyanates and nitriles reach the intestinal tract, which is the site of the action of dietary isothiocyanates, and act as protective agents (Getahun & Chung, 1999; Rungapamestry, Duncan, Fuller, & Ratcliffe, 2007; Shapiro, Fahey, Wade, Stephenson, & Talalay, 1998; Traka & Mithen, 2009). Cruciferous vegetables, however, are often eaten after boiling, steaming or microwaving, which affects the preservation of glucosinolate (Francisco, Velasco, Moreno, Garcia-Viguera, & Cartea, 2010) and the content of health-promoting polyphenols (Faller & Fialho, 2009). Furthermore, cooking and the thermal processing of

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cruciferous vegetables inactivate thioglucosidase, by which most glucosinolates move to the intestinal tract in an intact form. The metabolic fate of intact glucosinolates in the intestines and the involvement of intestinal microbiota are important issues remaining to be addressed. However, limited information is available on the metabolic fate of glucosinolates in the intestines. Glucosinolates have been reported to be hydrolyzed by bacterial thioglucosidase derived from intestinal microbiota or single strains of intestinal bacteria such as the genera *Bacteroides* (Krul et al., 2002) and *Bifidobacterium* (Cheng, Hashimoto, & Uda, 2004). In addition, sulfatase from intestinal microbiota may be involved in the digestion of glucosinolates, either alone or in combination with bacterial thioglucosidase. However, no studies addressing this issue have been reported thus far. Therefore, it is important to obtain further information on the digestibility of glucosinolates by the intestinal microbiota of mammals because the metabolic behavior of glucosinolates in the bowel is directly related to the bioavailability of glucosinolates or their digested products. In this study, we investigated the breakdown of desulfosinigrin along with sinigrin by the intestinal microbiota of rats and showed that desulfosinigrin can be the precursor of both nitriles, ACN and CETP.

2. Materials and methods

2.1. Chemicals

Sinigrin was purchased from Extrasynthese (Genay, France). Sulfatase from *Helix pomatia* (Type H-1) was purchased from Sigma-Aldrich Japan and used for the degradation of sinigrin and desulfosinigrin or the preparation of desulfosinigrin. ACN, allyl isothiocyanate (AITC), phenyl isothiocyanate, and *p*-nitrocatechol sulfate dipotassium salt dihydrate were purchased from Tokyo Chemical Industries, Co., Ltd. (Tokyo, Japan). Glucose-free M9 medium (pH 7.05) was prepared by dissolving 6.0 g of disodium hydrogen phosphate, 3.0 g of potassium dihydrogen phosphate, 5.0 g of sodium chloride, 1.0 g of ammonium chloride, 0.12 g of magnesium sulfate, and 4.0 mg of mg vitamin B_1 in 1.0 l of Milli-Q water. These and other analytical grade chemicals, including florisil for column chromatography, were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Purified desulfosinigrin and CETP were prepared as described below.

2.2. Gas chromatography–mass spectrometry analysis of ACN, AITC, and CETP

Gas chromatography–mass spectrometry (GC–MS) analysis was performed on a Hewlett–Packard 5971A GC–MS instrument, and GC separation was performed on a 30 m × 0.25 mm inner diameter (i.d.) Thermo Fisher Scientific TR-WAX column, using helium as the carrier gas. The column temperature was kept at 60 °C for 4 min and programmed to increase to 220 °C at 5 °C/min. The temperatures of the injection port and mass spectrometer were maintained at 250 °C and 170 °C, respectively. Identification of ACN (retention time: 5.85 min) and AITC (retention time: 10.84 min) was performed in accordance with both the recorded mass spectral data and GC retention times of their authentic specimens. CETP was identified by comparing its mass spectrum (Fig. 1) with that in the literature (Spencer & Daxenbichler, 1980) along with its retention behavior under the GC–MS conditions. The compound was detected at 22.75 min, considerably later than AITC, even though CETP and AITC have the same molecular formula, C_4H_5NS. The concentrations of ACN, AITC, and CETP in the sample extracts were determined by the ratios of their peak areas to the peak area of the internal standard (phenyl isothiocyanate). The ratios of authentic ACN and AITC and isolated CETP were determined beforehand by using known quantities under the same GC–MS conditions.

2.3. High performance liquid chromatography analysis of sinigrin and desulfosinigrin

High performance liquid chromatography (HPLC) analysis was carried out with a Hitachi L-6200 apparatus equipped with a Hitachi L-4250 UV–VIS detector and a Hitachi D-2500 Chromato-Integrator. The HPLC separation was performed on a Develosil C30-UG column (4.0 mm i.d. × 150 mm, Nomura Chemical Co. Ltd., Seto, Japan). The mobile phase was 4% aqueous methanol (Barbieri, Pernice, Maggio, De Pascale, & Fogliano, 2008), and had a flow rate of 0.6 ml/min. Sinigrin (retention time: 1.96 min) and desulfosinigrin (retention time: 10.01 min) were detected at 227 nm, and their concentrations were determined by comparing their peak areas with those of authentic specimens.

2.4. Preparation and purification of desulfosinigrin

Sinigrin (600 mg) dissolved in 150 ml of 0.05 M McIlvaine buffer (pH 6.0) was incubated with sulfatase (350 U) at 37 °C for 6 h. The reaction mixture was combined with 300 ml of ethanol (final concentration: 75%), and the resulting precipitate was removed by centrifugation at 2000 × *g* for 5 min. After ethanol was completely removed from the supernatant under vacuum, the residue was dissolved in a small volume of Milli-Q water and washed with diethyl ether to remove the volatiles produced during the incubation. The aqueous residue was passed through a diethylaminoethyl (DEAE)-Sephadex A25 column (10 mm × 80 mm) which had been washed with 0.5 M pyridine-acetate and equilibrated with water (Michinton, Sang, Burke, & Truscott, 1982). The column was rinsed with 30 ml of water, and the eluate was freeze-dried, yielding approximately 292 mg of white powder. Structural confirmation was performed by electrospray ionization (negative ion mode) mass spectrometry on a Micromass Quattro LC instrument at a cone voltage of 30 V, and the product was used as desulfosinigrin. Its purity was estimated to be over 99% by the HPLC described above.

2.5. Preparation and purification of CETP from defatted meal of yellow mustard seeds

Fifty grams of yellow mustard (*Brassica juncea* Czern. et Coss.) seed meal defatted with a mixture of hexane-diethyl ether (1:1) was autolyzed in 250 ml of 0.1 M McIlvaine buffer (pH 5.2) containing 2.5 mM ferrous sulfate for 90 min at an ambient temperature to produce CETP along with ACN and AITC. The autolysate was extracted with 300 ml of diethyl ether. After dehydration over anhydrous sodium sulfate, the extract was concentrated under a nitrogen stream and chromatographed on a florisil column (9 mm i.d. × 150 mm) prepared with hexane (Cole, 1975). After washing the column with hexane (50 ml), we performed stepwise elution with 50-ml portions of hexane and hexane-diethyl ether mixtures consisting of different ratios (4:1, 7:3, and 3:2). Isothiocyanates, including AITC and nitriles with the exception of CETP, were found in the hexane- and hexane-diethyl ether (4:1)-eluted fractions. The target compound, CETP, was contained in the hexane-diethyl ether (7:3) eluted fraction as the major component along with small amounts of 1-cyano-3,4-epithiobutane. This fraction was re-chromatographed on another florisil column (9 mm i.d. × 100 mm) using a hexane-diethyl ether mixture (85:15, 75:25, and 65:35) as the eluent. The hexane-diethyl ether (75:25)-eluted fraction was concentrated under a nitrogen stream to give 6.0 mg of pale yellow oil. The GC–MS analysis showed that the oil contained 92.6% CETP and 4.8% 1-cyanoepithio-3,4-butane with no AITC and ACN. The oil was dissolved in acetone to obtain a CETP concentration of 15 μg/μl, and then stored at −30 °C until use.

2.6. Animals and preparation of their intestinal microbiota

Five-week-old male Wistar rats (approximately 130 g) were purchased from Charles River Japan, Inc. (Yokohama, Japan). All