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Bioactivation of Selenocysteine Derivatives by β-Lyases Present in Common Gastrointestinal Bacterial Species

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Abstract: Studies in cell cultures and animal models have demonstrated cancer chemopreventive effects of certain selenium compounds. Here we describe the screening of cysteine *S*-conjugate β-lyase activity in bacterial species that are implicated in the bio-activation of sulfur- and selenocysteine derivatives.

We screened a range of bacterial species commonly found in the human intestine for β-lyase activity on Se-*p*-methoxybenzylselenocysteine and the natural occurring S-methylcysteine and Se-methylselenocysteine conjugates. A high-performance liquid chromatography (HPLC)-assisted assay was established to determine specific activities of each strain. Of the 29 tested bacterial species, 22 showed specific activities towards the test compound reaching up to 10.1 U/mg protein, thereby accounting for 75% of total fecal activity (13.3 U/mg protein).

Lysates of four bacterial strains *(Bacteroides distasonis, Bacteroides vulgatus, Enterococcus faecalis, and Enterococcus faecium),* which exhibited high specific activities towards the test compound and which are known to be present at high numbers in the human intestine, were characterized further. Our results indicate that β-lyase activity is widely distributed in human intestinal bacteria and might play a key role in the bioactivation of selenocysteine derivatives.

Key words: Selenium, cancer prevention, gut flora, intestine, bioactivation, beta-lyase, Se-methylselenocysteine

Introduction

In the last two decades there has been a considerable interest in the discovery of naturally occurring compounds with a high potential in cancer prevention [1, 2]. Colon cancer in particular, with nearly a half-million cases per year worldwide [3], is a major target in dietary cancer prevention.

Intervention studies such as the Nutritional Prevention of Cancer (NPC) trial have provided strong evidence for the efficacy of selenium as an anticancer agent [4]. This placebo-controlled trial showed a 50% reduction in total

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cancer incidence in a large, selenium-adequate U.S. population treated with 200 µg selenium as selenium-enriched yeast. Further analysis of the NPC trial on colorectal carcinoma incidence revealed a significant decrease in risk associated with selenium supplementation of subjects in the lowest tertile of baseline selenium and current smokers [5]. These results gave rise to a huge phase III study, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), designed to test the efficacy of selenium (200 µg selenomethionine) and vitamin E (400 mg DL-α-tocopherol), both alone and in combination, in the prevention of prostate cancer [6]. Studies in cell cultures and animal models have demonstrated cancer chemopreventive effects of certain selenium compounds [7–9]. Several studies have indicated that selenium and sulfur compounds display anticancer activities, with the former being effective to a much higher degree [2, 10]. Among these compounds there is a substantial number of sulfur- and especially selenium-containing derivatives of methionine and cysteine, which have been found in *Allium* and *Brassica* species [11–13]. In rats, selenium-enriched broccoli proved very effective in the prevention of chemically induced colon cancer [14].

Recent advances in analytical techniques revealed a number of new selenium-containing natural products discovered in selenium-enriched plants and yeasts [15]. Thus, garlic *(Allium sativum)* and selenium-enriched yeast *(Saccharomyces cervisiae)* exhibit a large number of cysteine- and selenocysteine-conjugates, such as S-methylcysteine, Se-methylselenocysteine (both Figure 1), and γ-glutamyl-Se-methylselenocysteine [13, 16–18].

Although it is suitable to describe effects of Se in terms of the element, it must always be kept in mind that chemical form and dose are determinants of its biological activity as a cancer-preventive agent or toxicant. Certain selenium metabolites such as methylselenol (MeSeH) have potent anticancer activity [7, 19–21]. MeSeH can be formed by methylation of H_2Se, directly liberated from selenomethionine by cystathionine γ-lyase [22] or generated from Se-methylselenocysteine by β-lyases [23], which are present in the kidney, liver, or the gastrointestinal tract and various bacteria therein [24–27]. Other enzymes can also catalyze β-lyase reactions as has been shown for cystathionine γ-lyase [28] and β-lyases/glutamine transaminase K [29]. Thus, the bacterial β-lyases might represent key enzymes for the gastrointestinal conversion and bioactivation of dietary-derived S- and Se-conjugates. The first respective general overview on β-lyase activity present in gastrointestinal bacteria was published by Larsen [24]. Out of 43 tested intestinal bacteria, only 27 showed β-lyase activity and only members of the genus *Bacteroides* and the species *Clostridium ramosum, Peptostreptococcus productus,* and *Streptococcus faecalis*

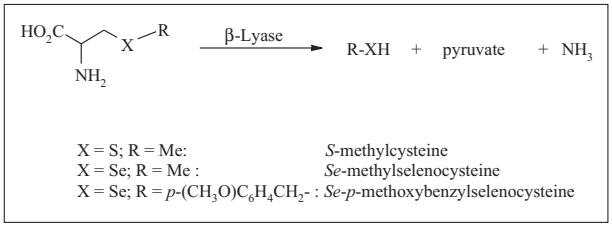

Figure 1: β-Elimination of S- and Se-cysteine conjugates catalyzed by β-lyases.

have been reported to be found frequently in high numbers in human fecal samples [30–32]. However, *Eubacterium limosum,* which has been reported to exhibit the highest β-lyase activities, is not a common member of the human gut microbial community [33]. Therefore, it is hard to delineate the importance of the gastrointestinal bacteria for MeSeH generation *in vivo*.

Using Se-*p*-methoxybenzylselenocysteine (Se-MBS) (Figure 1) as a substrate, we established a high-performance liquid chromatography (HPLC)-based screening assay for β-lyase activities of gastrointestinal bacteria known to be present in high numbers in human fecal samples, such as members of the genera *Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Eubacterium,* and *Lactobacillus* [30–35]. Furthermore we investigated substrate specificity of four highly active bacterial strains with two physiologically relevant, naturally occurring S- and Se-cysteine conjugates. Our results point to the importance of bacterially-derived enzymes for the bio-conversion of dietary-derived precursors to chemopreventive agents such as MeSeH.

Materials and Methods

Chemicals and synthesis

Selenocysteine was purchased from Euburon Chemicals (Belgium). S-methylcysteine was purchased from Aldrich (Germany). Se-*p*-methoxybenzylselenocysteine (Se-MBS) and Se-methylselenocysteine were synthesized from selenocysteine according to procedures reported previously [16, 36]. ^{1}H NMR and ^{13}C NMR spectra of the synthesized compounds were recorded at 300 MHz on a Bruker AMX 300 with Me_4Si (δ 0) as the internal standard.

Organisms and culture conditions

All reference strains used in this study were obtained from the sources indicated (DSMZ, Deutsche Sammlung von

Mikroorganismen und Zellkulturen, Braunschweig, Germany; IFM, Institute for Microecology, Herborn, Germany; ATCC, American Type Culture Collection, Rockville, MD, USA). All IFM strains are human fecal isolates from our laboratory and were identified using the Vitek® System (bioMérieux, Nuertingen, Germany). All strains were cultured at 37°C in ST-medium (ST) under strictly anoxic conditions with N_2/CO_2 (80:20, v/v) as gas phase [37, 38], which contained per liter: 9 g tryptically digested peptone from meat, 1 g proteose peptone, 3 g meat extract, 4 g yeast extract, 6 g glucose, 3 g NaCl, 2 g Na_2HPO_4, 0.5 mL Tween 80, 0.25 g L-cystine, 0.25 g L-cysteine • HCl, 0.1 g $MgSO_4 \cdot 7\ H_2O$, 5 mg $FeSO_4$ ($7\ H_2O$, 3.4 mg $MnSO_4 \cdot 2\ H_2O$, pH 7.

Preparation of cell free extracts

The cell and fecal sample extracts were prepared in the presence of oxygen at 4°C from cultures grown overnight in ST medium or fresh feces. The samples were centrifuged (10 000 × g, 15 minutes), washed twice with 100 mM potassium phosphate buffer (pH 7.0), re-suspended in the same buffer supplemented with DNase, and ruptured by twofold passage through a French pressure cell at 130 MPa (Aminco, Silver Springs, USA). Cell-free extracts were obtained by centrifugation at 14 000 × g for 20 minutes at 4°C.

Enzyme activity

The specific β-lyase activity was determined by following pyruvate formation [39]. Se-*p*-methoxybenzylselenocysteine, S-methylcysteine, and Se-methyl-selenocysteine, respectively, were incubated at various concentrations (10 μM–0.9 mM) with bacterial lysates in 50 mM sodium borate buffer at 37°C and pH 8.6, which is the optimal pH for cytosolic β-elimination reactions [40]. The incubation volume was 100 μL. After 20 minutes, the reaction was stopped with 500 μL of 14% *o*-phenylenediamine in 3 N HCl. Following heating for 45 minutes at 60°C, the amount of derivatized pyruvate was analyzed by HPLC. Incubations without the cell-free extract and without substrate, respectively, were used to correct for spontaneous and non β-lyase-mediated degradation. Protein content was determined as described by Bradford [41].

Analysis of pyruvate formation from *Se*- and *S*-conjugates by HPLC

Fifty μL of derivatized incubation mixture were analyzed using an HPLC system which consisted of a L-6200A pump, a L-7480 fluorescent detector, an AS-2000A autosampler (all Merck-Hitachi, Darmstadt, Germany) with a 100-μL sample loop, a solvent degasser, and a 125 × 4 mm, 4 μm-particle-sized RP-18 column (Sepserv, Berlin). The mobile phase consisted of water and methanol (60:40, v:v) and the flow was set to 0.5 mL/minute. Pyruvate formation was calculated using an external calibration curve. Derivatized pyruvate eluted at 12.65 minutes and was detected with excitation at 336 nm and emission at 420 nm.

Results and Discussion

In the present study we analyzed β-lyase activities of several bacterial species present in the human intestinal tract. Se-MBS has been shown to be a good substrate for renal β-lyase, therefore it was chosen as test substrate [36]. Since β-lyase activity of *Eubacterium limosum* has been extensively studied [42], we obtained exemplary kinetic data with *Eubacterium limosum* as a criterion strain. Enzyme activity followed Michaelis-Menten kinetics. We obtained K_m (1.6 μM) and V_{max} (56.5 U/ mg protein) values; however we suppose that more than one enzyme can possibly exhibit β-lyase activity and thus Michaelis-Menten constants are not suitable to describe the β-lyase activity of the bacterial lysates used in this study. Since *Eubacterium limosum* is not present in high numbers in human fecal samples [33], it is unlikely that this organism plays a major role in the intestinal activation of selenocysteine compounds.

The same applies for most of the strains that were used by Larsen since they are not commonly found in high numbers in the gut [24]. Therefore, we extended the given list to numerically more important species. Our main focus was on members of the genera *Bacteroides, Bifidobacterium, Clostridium, Enterobacteriaceae, Eubacterium,* and *Lactobacillus* which have been reported to be present abundantly in human feces [30, 32, 33, 43, 44].

Considerable β-lyase activities for Se-MBS were found in 22 of 29 tested bacterial strains and in fresh feces, indicating a general distribution of β-lyases among gastrointestinal bacteria, as reported previously [24]. High levels of β-lyase activity (> 1 U/mg protein) were detected only in 10 of the 30 strains and in fresh feces (Table I). In general, the highest β-lyase activity was found among species of the genera *Bacteroides* and *Enterococcus* (Table I).

To decide whether these bacteria might be important for bio-conversion of dietary-derived chemopreventive substances, all four bacterial species showing the highest β-lyase activities *(Bacteroides distasonis, B. vulgatus, Enterococcus faecalis, E. faecium)* were further tested with S-methylcysteine and Se-methylselenocysteine. Both compounds are major natural ingredients of garlic *(Alli-*

Table 1: Specific β-lyase activities for Se-(4-methoxybenzyl)-L-selenocysteine in intestinal bacteria and their numerical occurrence in human fecal samples

Species	Strain	β-lyase activity U/mg protein (S.D.)	Range (mean)[a]	Reference to bacterial occurrence
Total bacteria		13.3 (± 3.6)	11.2–11.4 (11.3)	[33, 48]
Bacteroides distasonis	DSM 20701	9.2 (± 0.07)	9.3–12.5 (10.6)	[30, 48, 49]
Bacteroides sp.(B. fragilis)	IFM	1.7 (± 1)	7.5–11.8 (10.3)	[30]
Bacteroides sp. (B. vulgatus)	IFM	7.4 (± 4)	8.6–13.5 (10.5)	[32]
Bacteroides thetaiotaomicron	DSM 2079	2.8 (± 1)	6.3–12 (10.3)	[49]
Bifidobacterium adolescentis	ATCC 15703	0.65 (± 0.18)	5.7–13.4 (10.3)	[30]
Bifidobacterium catenulatum	ATCC 27539	0.43 (± 0.02)	[b]	[50]
Bifidobacterium infantis	ATCC 15697	0	6.1–12.4 (9.9)	[30]
Bifidobacterium longum	ATCC 15707	0.32 (± 0.03)	9.1–11.3 (10.4)	[30]
Clostridium acetobutylicum	ATCC 824	0	7.6	[30]
Clostridium barati	DSM 601	0.05 (± 0.05)	5.4–8.7 (7.1)	[30]
Clostridium butyricum	DSM 10702	0	10.4	[30]
Clostridium cellobioparum	DSM 1351	0.03 (± 0.03)	5.7–6.5 (6.1)	[30]
Clostridium clostridiforme	DSM 933	0.10 (± 0.05)	9.6–11.1 (10.3)	[30]
Clostridium difficile	DSM 1296	0.60 (± 0.08)	4.8	[30]
Clostridium innocuum	DSM 1286	0.09 (± 0.0)	n.d.[c]	[43]
Clostridium pasterianum	DSM 525	0.24 (± 0.03)	10.0	[30]
Clostridium perfringens	DSM 756	0.08 (± 0.07)	3.8–12.5 (6.9)	[30]
Clostridium sartagoforme	DSM 1292	1.01 (± 0.76)	8.3–10.9 (9.6)	[30]
Clostridium sordellii	DSM 2141	4.01 (± 1.67)	9.9	[30]
Clostridium sporosphaeroides	DSM 1294	0	5.5–10.9 (8.4)	[30]
Enterococcus sp. (E. faecalis)	IFM	10.1 (± 2.43)	5–6 (5.5)	[44]
Enterococcus sp. (E. faecium)	IFM	9.32 (± 2.27)	5–6 (5.5)	[44]
Eubacterium barkeri	ATCC 25849	0.10 (± 0.05)	n. d.	[33]
Eubacterium biforme	DSM 3989	0	7.59–9.1 (8.35)	[33]
Eubacterium limosum	DSM 20543	4.24 (± 0)	n.d.	[33]
Eubacterium moniliforme	DSM 3984	2.44 (± 0.06)	n.d.	[33]
Eubacterium multiforme	DSM 20694	0	n.d.	[33]
Eubacterium rectale	ATCC 33656	0	5.7–11.6 (9.4)	[33]
Lactobacillus fermentum	DSM 20052	0	3.6–11.5 (8.3)	[30]

[a] Range and mean count of bacteria expressed as number of organisms log10 per gram feces (dry weight in persons who consumed Western diet), [b] no direct counts given, [c] not detected. Data are expressed as mean ± SD from four individual experiments

Table II: Specific β-lyase activities for Se-methyl-L-cysteine and S-methyl-L-cysteine for most abundant bacterial species

Species	Specific β-lyase activity[a] (U/mg)	
	Se-methyl-L-cysteine (± SD)	S-methyl-L-cysteine (± SD)
Bacteroides distasonis	0.3 (0.05)	0.3 (0.04)
Bacteroides vulgatus	0.9 (0.09)	0.3 (0.05)
Enterococcus faecalis	1.3 (0.05)	1.1 (0.05)
Enterococcus faecium	1.2 (0.08)	1.9 (0.08)

[a] substrate concentrations used were 100 μM. Data are expressed as mean ± SD from four individual experiments

um sativum), selenium-enriched yeast and broccoli [45, 46]. Our results indicate that all four bacteria tested displayed a high β-lyase activity for these physiologically important compounds (Table II). Since we did not isolate a purified β-lyase, we did not determine K_m and V_{max} values.

Amino acids are mainly absorbed in the small intestine via sodium-dependent transporters. As both *Enterococcus* species are mainly found in the human small intestine [47], they may play a key role in the bio-activation of sulfur- and selenocysteine-derivatives from the diet *in vivo*.

In summary, we were able to show that numerically important bacteria from the human intestinal tract exhibit high β-lyase activity on S- and Se-containing compounds. The contribution of the gastrointestinal bacteria to the chemopreventive effects of selenium-enriched yeast ob-

served in the NPC trial might have been crucially important. It remains to be elucidated whether shifts in the amount and pattern of intestinal bacteria affects the bioconversion of dietary-derived substrates for β-lyase. Inter-individual variations in the composition of the intestinal flora may lead to a differentiated response to cancer protective food (i.e. broccoli and garlic). However, given the high capacity of the β-lyases expressed by the abundant intestinal bacteria, it appears safe to conclude that the enzymatic conversion of chemopreventive precursors of e.g. MeSeH is not limiting in a healthy human gastrointestinal tract.

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