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In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora

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Abstract

Stevia mixture, sweeteners extracted from the leaves of *Stevia rebaudiana* Bertoni, consists mainly of stevioside and rebaudioside A (glycosides of the diterpene derivative steviol). The aim of this study was to investigate human intestinal metabolism of stevia mixture and its α-glucose derivative (known in Japan as enzymatically modified stevia) by LC/MS/ESI analysis. Degradation was examined by incubating stevia mixture, enzymatically modified stevia, stevioside, rebaudioside A, α-monoglucosylstevioside, α-monoglucosylrebaudioside A and the aglycone, steviol with pooled human faecal homogenates (obtained from five healthy volunteers) for 0, 8 and 24 h under anaerobic conditions. Stevia mixture, enzymatically modified stevia, stevioside and rebaudioside A (0.2 mg/ml) were completely eliminated within 24 h, whereas no degradation of steviol (0.08 and 0.2 mg/ml) appeared to be found during the incubation period. Stevia mixture, stevioside and rebaudioside A appeared to be hydrolyzed to steviol by human intestinal microflora: this observation is consistent with previous rat metabolism studies. Similarly, enzymatically modified stevia appeared to be metabolized via stevia components and, finally, to steviol. This study suggests that there are apparently no species differences in intestinal metabolism of stevia mixture between rats and humans.
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Keywords: Stevia mixture; Enzymatically modified stevia; Steviol; LC/ESI/MS; Human intestinal microflora

1. Introduction

Stevia rebaudiana Bertoni, belonging to the *Compositae* family, is a sweet herb native to Brazil and Paraguay. Stevia sweeteners, extracts from the leaves of this herb, are commercially available in Japan, Korea, China, South-East Asia and South America, where they have been used for some decades to sweeten a variety of foods including beverages, confectionery, pickled vegetables and seafoods. Recently, stevia extracts have been extensively used as the dietary supplements in USA.

Stevia sweeteners are glycosides of the diterpene derivative steviol (*ent*-13-hydroxykaur-16-en-19-oic acid), consisting mainly of stevioside and rebaudioside A, together with the other components rebaudioside C and dulcoside A plus the extremely minor components rebaudioside B, D and E and steviolbioside. These chemicals account for approximately 5–10% of dry leaf weight. They have sweetening potentials 200–300 times that of sucrose and are thermally stable (Soejarto et al., 1982; Hanson and De Oliveira, 1993). The α-glucose additive of stevia, α-glucosyltransferase-treated stevia (enzymatically modified stevia) gives improved taste quality, and is widely used in Japan (Sennma et al., 2001).

The toxicity of stevioside has been investigated extensively (Mori et al., 1981; Aze et al., 1991; Xili et al., 1992; Toyoda et al., 1995, 1997; Matsui et al., 1996a,b; Toskulkao et al., 1997): results of short- and long-term

Abbreviations: IS, internal standard; LC/MS/ESI, liquid chromatography/mass spectrometry/electrospray ionization; Rt, retention time.

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toxicity, carcinogenicity and genotoxicity studies suggest that stevioside shows no serious toxicity to mammalian species. Metabolism and elimination of stevioside has been studied in rats (Nakayama et al., 1986; Cardoso et al., 1996). In vivo studies indicate that stevioside is hydrolyzed to steviol (the aglycone) in the caecum then absorbed. In the same manner, stevioside and rebaudioside A are degraded, ultimately to steviol, by rat intestinal microbial cells under anaerobic conditions (Wingard et al., 1980). However, although the metabolic pathway in the rat is well known, human intestinal metabolism of stevioside and its related compounds has still not been characterized. In this study, we investigate the metabolism of stevia-related compounds (including enzymatically modified stevia) using pooled human faecal specimens and liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandern mass spectrometry (LC/MS/MS) analyses.

2. Materials and methods

2.1. Chemicals

Stevia mixture (main components: rebaudioside A, stevioside, rebaudioside C, dulcoside A; Fig. 1), enzymatically modified stevia (main components: α-glucosylrebaudioside A, α-glucosylstevioside, α-glucosylrebaudioside C, α-glucosyldulcoside A; Fig. 2), rebaudioside A, stevioside, steviol, rebaudioside C, dulcoside A, rebaudioside B, rubusoside, α-monoglucosylrebaudioside A (main components: α-monoglucosylrebaudioside A-1 85.9%, α-monoglucosylrebaudioside A-2 + A-3 10.7%; Fig. 2) and α-monoglucosylstevioside (main components: α-monoglucosylstevioside-1 + -2 52.7%, α-diglucosylstevioside 32.9%; Fig. 2) were kindly provided by the Japan Stevia Industrial Association (Tokyo, Japan). Abietic acid was purchased from Wako Pure Chemical (Osaka, Japan). All other reagents were of the highest purity commercially available, or of HPLC grade.

2.2. Human faecal homogenate preparation

Faecal samples were obtained from five healthy Japanese male volunteers (29–34 years old) on the mornings of the stevia mixture and enzymatically modified stevia experiments, at the Yakult Central Institute for Microbiological Research (Tokyo, Japan). After the purpose of this study was explained to the volunteers, all gave written informed consent to the protocol. Volunteers were required not to take synthesized sweeteners or drugs for 3 days before sample collection. Table 1 shows the results of microbiological examination of faeces obtained from the volunteers before experimental anaerobic incubation. The values obtained from the faecal specimens fell within the range of data previously reported by Mitsuoka (1990) and Finegold et al. (1974). The two sets of results with microbiological examinations, for stevia and enzymatically modified stevia studies, were similar (not significantly different by Student's *t*-test). For the preparation of faecal homogenates, fresh faecal samples were weighed and homogenized with 5 vols (w/v) of 0.2 M potassium phosphate buffer (pH 7.0) by vigorous shaking. The homogenates were centrifuged at 500 *g* for 1 min at 4 °C. Identical volumes of supernatants obtained from five samples were gathered to prepare a 25-times diluted pooled human faecal suspension, which was then diluted further for experimental use. All processes were performed under anaerobic conditions.

2.3. Faecal homogenate incubation and extraction

Using a glass tube, reaction mixtures were prepared containing 50-times diluted volumes of pooled human faecal homogenates, together with test mixture (0.2 or 10 mg/ml stevia mixture, rebaudioside A, stevioside, α-monoglucosylrebaudioside A or α-monoglucosylstevioside or 0.08 or 0.2 mg/ml steviol), 0.1 M potassium phosphate buffer (pH 7.0) and 1 (v/v)% dimethyl sulfoxide in a total volume of 2 ml. These reaction mixtures were then incubated at 37 °C for 0, 8 and 24 h under anaerobic conditions. The reaction was stopped by the addition of 2 ml of acetonitrile. The suspended faecal samples were frozen and stored at −80 °C until analysis.

After thawing the incubated samples at room temperature, 1 ml of acetonitrile and 25 μl of abietic acid in methanol (as an internal standard) were added to 200 μl of the faecal samples. The mixtures were centrifuged at 1620 *g* for 10 min at 10 °C, and the supernatants were evaporated under a nitrogen flow. To the residues, 200 μl of 30 (v/v)% acetonitrile aqueous solution was added and then vortex-mixed. Samples were then injected onto the LC/MS systems as described below.

2.4. LC/MS conditions for stevia and enzymatically modified stevia analyses

The samples were separated on a Mightysil RP-18 GP column (250 × 4.6 mm, 5 μm; Kanto Chemical, Tokyo, Japan) by a gradient solvent system consisting of acetonitrile and 10 mM ammonium acetate at a flow rate of 0.8 ml/min. The percentage of acetonitrile was increased from 30 to 85% over 33 min. After 33 min, the column was re-equilibrated with the initial mobile phase for 10 min. The standard solutions of parent compounds and their metabolites were introduced to the mass spectrometer using the flow injection analyses method. LC/MS was carried out by coupling a Hewlett Packard HPLC

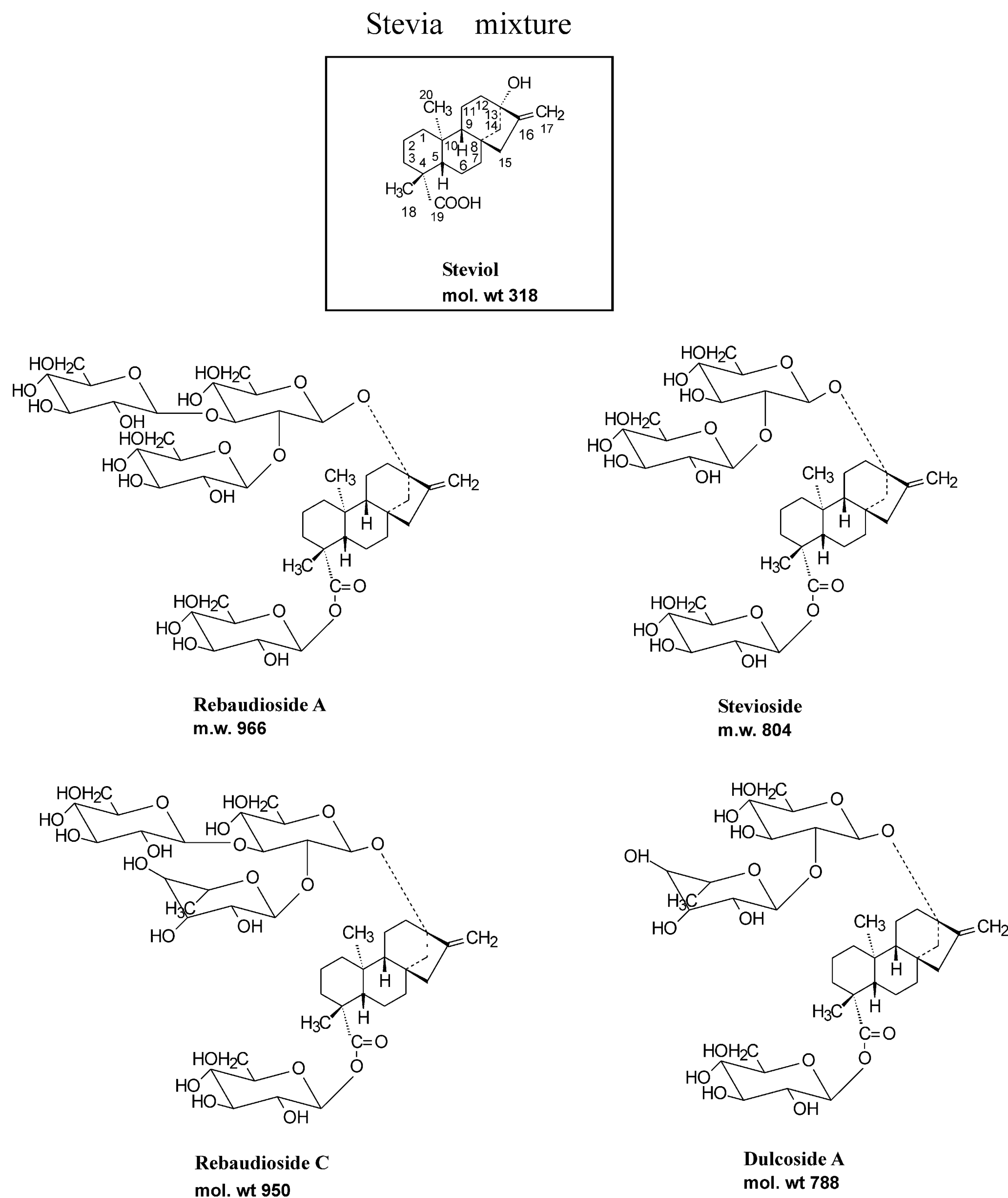

Fig. 1. Chemical structures of stevia mixture.

system (HP-1050) to a Finnigan TSQ-7000 ion trap mass spectrometer (Thermo Finnigan, San Nose, CA, USA). LC/ESI/MS was performed on the mass spectrometer operated in both positive and negative ion detection modes. The samples were detected by operating the mass spectrometer either in the full scan mode or by selected ion monitoring of the pseudo-molecular ions. MS/MS of fragment ions on the TSQ-7000 mass spectrometer were obtained with −25 V and +40−50 V collision energy in the positive and negative ion modes, respectively.

3. Results

3.1. Structual identification by LC/ESI/MS and LC/MS/MS analyses

Chemical structures of parent compounds, stevia mixture and enzymatically modified stevia consist of the aglycone, steviol and more than one mole of D-glucose or L-rhamnose (Fig. 1). Identification of metabolites of stevia mixture and enzymatically modified stevia incubated with human faecal homogenates was achieved

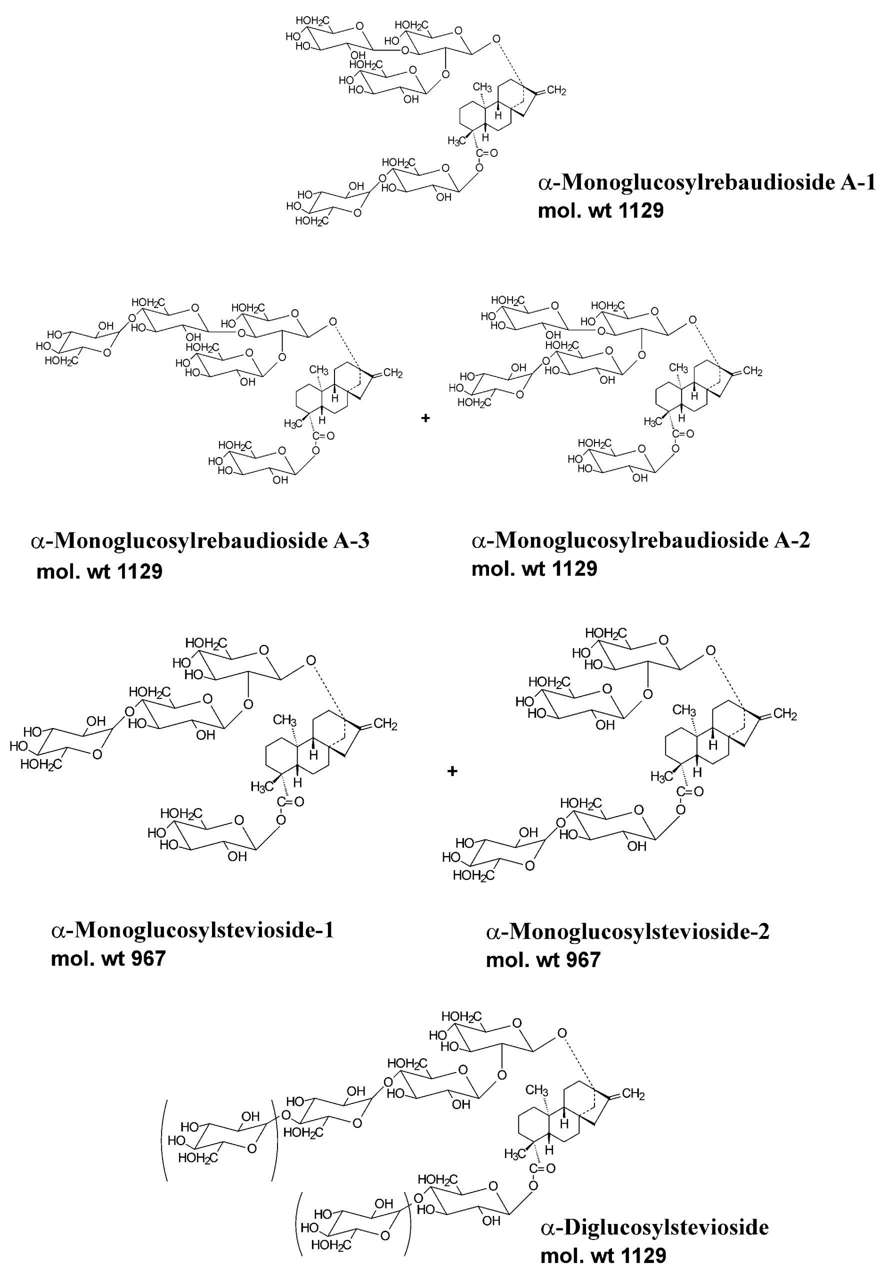

Fig. 2. Chemical structures of enzymatically modified stevia.

using HPLC retention times (Rt) and, LC/ESI/MS and MS/MS fragmentation (Table 2, Fig. 3), and was confirmed by comparative analysis with the parent compound and its metabolite standards.

Mass spectral analyses of stevia-related compounds were performed in positive and negative ESI ion modes. Full scan mass spectra for stevioside, rebaudioside A, dulcoside A, rebaudioside B, rubusoside, α-monoglucosylstevioside and α-monoglucosylrebaudioside A showed pseudo-molecular ions as ammonia additives ($[M+NH_4]^+$) in the positive ion mode. Furthermore, precursor ion ($[M+NH_4]^+$) scans for these compounds fragmented and generated a common product ion of *m/z* 319. On the other hand, no ion peak was found in the mass spectra (positive ion mode) for steviol, SG(1) and SG(2), the chemical structures of which contain 0 or 1 mol of glucose, respectively. However, the full scan mass spectra for all compounds including abietic acid (IS) showed pseudo-molecular ions as $[M–H]^-$ and $[M–H+CH_3COOH]^-$ in the negative ion mode. The characteristic mass spectrum for steviol showed a pseudo-molecular ion of *m/z* 635 as $[2M–H]^-$, which was not observed for other compounds. These results demonstrate that the structural determination of stevia related compounds and their

Table 1
Results of microbiological examination in faeces obtained from each five healthy male volunteers in stevia mixture and enzymatically modified stevia studies

Volunteer	Age (years)		(Unit: CFU/g fecal weight) Total bacteria	*Bifidobacterium*	*Bacteroides*	*Clostridium perfringens*	*Lactobacillus*	*Echerichia coli*	*Streptococus*	*Staphylococcus enterococcus*	*Pseudomonaseae*	Yeast (fungi)
		Medium	VLM	MPN	VLM	CW agar base	modified LBS	DHL	Columbia agar base	*Staphylococcus*	NAC	Chromo agar Candida
		Incubation condition	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Stevia mixture												
A	31		7.35×10^{10}	1.01×10^{10}	2.30×10^{10}	–	2.86×10^{6}	5.46×10^{7}	2.46×10^{6}	N.D.	N.D.	N.D.
B	29		4.75×10^{10}	5.75×10^{9}	8.50×10^{9}	3.50×10^{3}	5.28×10^{6}	1.90×10^{5}	3.76×10^{5}	N.D.	N.D.	N.D.
C	31		1.49×10^{10}	3.00×10^{9}	6.40×10^{9}	–	5.60×10^{4}	3.26×10^{7}	9.40×10^{6}	1.56×10^{5}	N.D.	N.D.
D	30		1.33×10^{10}	7.45×10^{9}	6.15×10^{9}	–	5.80×10^{3}	3.58×10^{5}	2.92×10^{5}	N.D.	N.D.	N.D.
E	37		7.05×10^{10}	8.05×10^{9}	3.10×10^{10}	–	3.14×10^{6}	8.80×10^{6}	2.24×10^{6}	N.D.	N.D.	N.D.
Mean	31.6		4.39×10^{10}	6.86×10^{9}	1.50×10^{10}	–	2.27×10^{6}	1.93×10^{7}	2.95×10^{6}	–	–	–
SD	3.1		2.91×10^{10}	2.65×10^{9}	1.13×10^{10}	–	2.25×10^{6}	2.38×10^{7}	3.74×10^{6}	–	–	–
Enzymatically modified stevia												
A	32		7.70×10^{10}	1.39×10^{10}	2.40×10^{10}	N.D.	1.02×10^{4}	6.80×10^{7}	6.80×10^{6}	N.D.	N.D.	N.D.
B	30		2.30×10^{10}	5.35×10^{9}	1.75×10^{9}	N.D.	4.34×10^{7}	2.64×10^{6}	2.60×10^{6}	N.D.	N.D.	N.D.
C	34		3.40×10^{10}	8.85×10^{9}	7.80×10^{9}	N.D.	2.80×10^{6}	1.24×10^{8}	1.10×10^{6}	2.04×10^{4}	N.D.	N.D.
D	31		5.70×10^{10}	1.65×10^{9}	1.50×10^{9}	N.D.	3.04×10^{4}	1.10×10^{6}	2.00×10^{2}	N.D.	N.D.	N.D.
E	37		7.25×10^{10}	5.85×10^{9}	2.35×10^{10}	N.D.	2.70×10^{5}	7.20×10^{7}	8.80×10^{5}	N.D.	N.D.	N.D.
Mean	32.8		5.27×10^{10}	1.01×10^{10}	1.44×10^{10}	–	9.30×10^{6}	5.35×10^{7}	2.28×10^{6}	–	–	–
SD	2.8		2.36×10^{10}	4.94×10^{9}	9.73×10^{10}	–	1.91×10^{7}	5.21×10^{7}	2.70×10^{6}	–	–	–

N.D. $<2 \times 10^{2}$.

Table 2
LC/ESI/MS and MS/MS fragmentation for related compounds of stevia mixture and enzymatically modified stevia and abietic acid (internal standard)

Compound	Mol. wt	Chemical* structure (a)	(b)	Substrate	Retention time (min)	Quasai-molecual ion (m/z) Positive ion $[M+H]^+$	$[M+NH_4]^+$	Negative ion $[M-H]^-$	$[M-H+CH_3COOH]^-$	$[2M-H]^-$	MS/MS Product ion (m/z), (precursor ion: $[M+NH_4]^+$) $[SH2G+H]^+$	$[3G]^+$**	$[SHG+H]^+$		$[2G]^+$**	$[S2H+H]^+$		[G]**	
Substrates or metabolites																			
α-Monoglucosyl-rebaudioside A (1, 2 or 3)	1129	i, j or f	b or c	α-MRA, Modified stevia	8.1	N.D.	1146	1128	1187	–	N.D.	487	N.D.		325	319	N.D.	163	N.D.
α-Monoglucosyl-rebaudioside A (1, 2 or 3)	1129	i, j or f	b or c	α-MRA, Modified stevia	8.3	N.D.	1146	1128	1187	–	N.D.	487	N.D.		325	319	N.D.	163	N.D.
α-Diglucosyl-stevioside	1129	k	a or e	α-MSV, Modified stevia	7.8	N.D.	1146	1128	1187	–	N.D.	487	N.D.		325	319	N.D.	N.D.	145
α-Monoglucosyl-stevioside (1 or 2)	967	c or h	a or e	α-MSV, Modified stevia	7.6	N.D.	984	965	1025	–	N.D.	487	N.D.		325	319	289	163	N.D.
α-Monoglucosyl-stevioside (1 or 2)	967	c or h	a or e	α-MSV, Modified stevia	8.4	N.D.	984	965	1025	–	N.D.	N.D.	481		325	319	N.D.	163	N.D.
Rebaudioside A	967	f	a	RA, Stevia, α-MRA, Modified stevia	9.2	N.D.	984	965	1025	–	643	487	481	N.D.	325	319	289	163	N.D.
Stevioside	804	c	a	SV, Stevia, α-MRA, Modified stevia	9.4	N.D.	822	803	863	–	643	N.D.	481	N.D.	325	319	289	163	145
Rebaudioside C	950	g	a	Stevia, Modified stevia	10.7	N.D.	968	949	1009	–	627	471	465	447,429	309,325	319	273	147	129
Dulcoside A	788	d	a	Stevia, Modified stevia	11.2	N.D.	806	787	847	–	627	N.D.	465,481	447,429	309,325	319	273	147	129
Steviol	318	H-	H-	SV, RA, Stevia, Steviol, α-MRA, α-MSV, Modified stevia	22.1	N.D.	N.D.	317	377	635	–	–	–	–	–	–	–	–	–
Internal standard																			
Abietic acid	302	***	–	31.1	N.D.	N.D.		301	361		–	–	–	–	–	–	–	–	–

(*continued*)

Table 2 (*continued*)

Compound	Mol. wt	Chemical* structure		Substrate	Retention time (min)	Quasai-molecual ion (m/z)					MS/MS Product ion (m/z), (precursor ion: $[M+NH_4]^+$)								
						Positive ion		Negative ion											
		(a)	(b)			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M-H+CH_3COOH]^-$	$[2M-H]^-$	$[SH2G+H]^+$	$[3G]^+$**	$[SHG+H]^+$		$[2G]^+$**	$[S2H+H]^+$		[G]**	
Metabolites																			
Rebaudioside B	804	f	H-	RA, Stevia, α-MRA, Modified stevia	8.6	N.D.	822	803	863	–	N.D.	487	481	N.D.	325	319	289	163	145
Rubusoside	642	a	H-	SV, RA, Stevia, α-MRA, α-MSV, Modified stevia	12.9	N.D.	660	641	701	–	N.D.	N.D.	481	N.D.	N.D.	319	273	163	145
											MS/MS Product ion (m/z), (precursor ion: $[M-H+CH_3COOH]^-$)								
													$[SHG-H]^-$			$[S2H-H]^-$			
SG(1)	480	a	H-	SV, RA, Stevia, α-MRA, α-MSV, Modified stevia	14.1	N.D.	N.D.	479	539	–			479			317			
SG(2)	480	H-	a	SV, RA, Stevia, α-MRA, α-MSV, Modified stevia	17.8	N.D.	N.D.	479	539	–			–			317			

SV: stevioside, RA: rebaudioside A, stevia: stevia mixture, α-MSV: α-monoglucosylstevioside, α-MRA: α-monoglucosylrebaudioside A, modified stevia: enzymatically modified stevia.

*, **, *** Chemical structures are shown in Fig. 3.

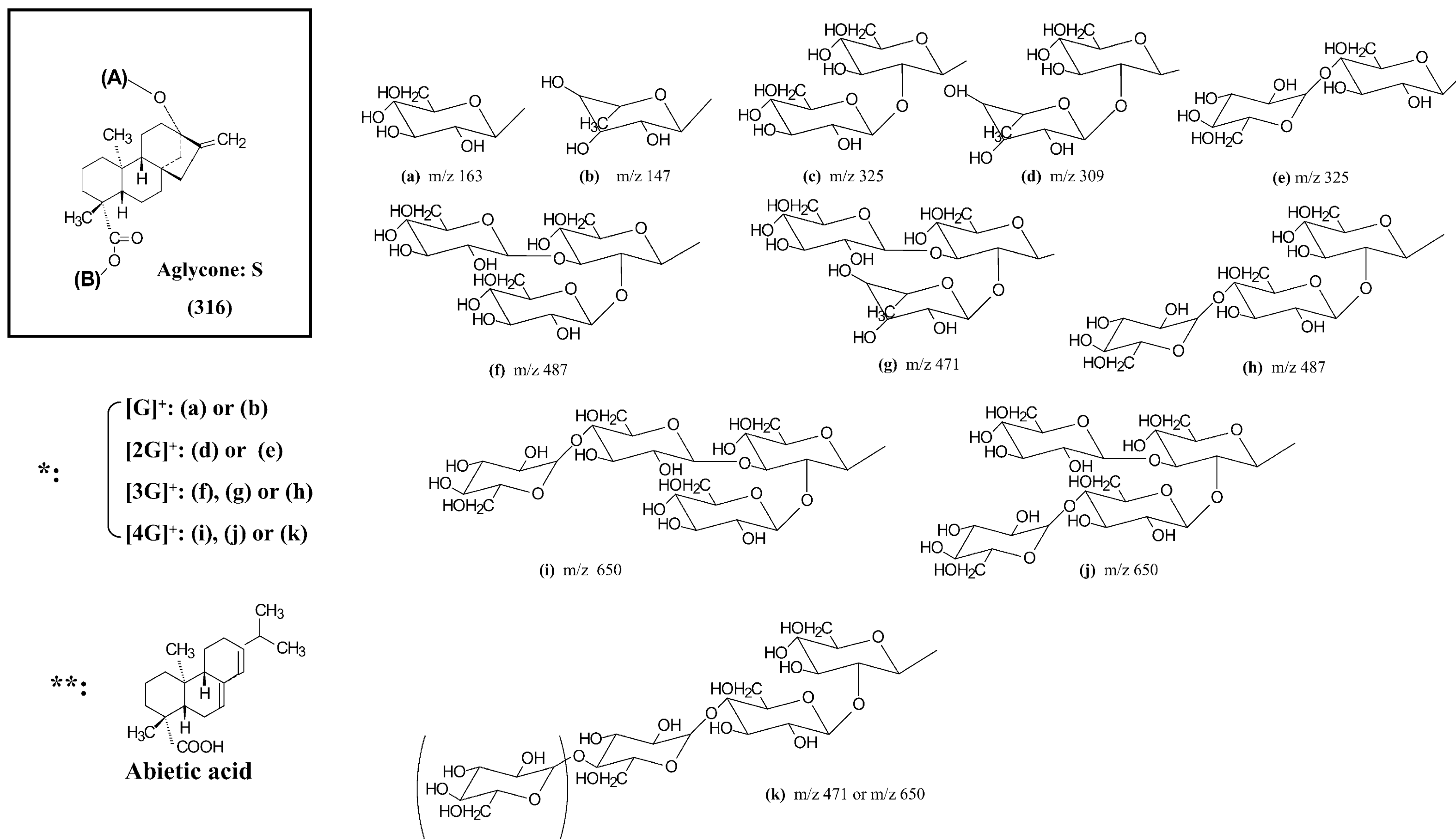

Fig. 3. Chemical structures and main fragment ions of related compounds of stevia mixture, enzymatically modified stevia and abietic acid (IS) (attached to Table 2).

metabolites in faecal specimens should be performed by the negative ion LC/ESI/MS method.

3.2. Stevioside

Negative ESI/MS full scan and monitor ion scan mass spectra were obtained from stevioside anaerobically incubated with pooled human faecal homogenates for 0, 8 and 24 h. Monitor ion peaks at *m/z* 863 (Rt: 9.4 min), *m/z* 701 (Rt: 12.9 min), *m/z* 539 (Rt: 14.1 and 17.8 min) and *m/z* 317 (Rt: 22.1 min) were identified on the monitor ion chromatograms throughout the incubation period, but no other eluted metabolite peak appeared on the full scan chromatograms. The Rt and fragmentation patterns observed in positive and/or negative ion ESI modes for these peaks were consistent with the standards of stevioside, rubusoside, SG(1), SG(2) and steviol (Table 2).

The proposed scheme for biotransformation of stevioside, based on the identification of human intestinal metabolites, is shown in Fig. 4.

3.3. Rebaudioside A

Negative ESI/MS full scan and monitor ion scan mass spectra were obtained from rebaudioside A anaerobically incubated with pooled human faecal homogenates for 0, 8 and 24 h. Monitor ion peaks at *m/z* 1025 (Rt: 9.2 min), *m/z* 863 (Rt: 8.6 and 9.4 min), *m/z* 701 (Rt: 12.9 min), *m/z* 539 (Rt: 14.1 and 17.8 min) and *m/z* 317 (Rt: 22.1 min) were detected on the monitor ion chromatograms, but no other eluted metabolite peak appeared was found on the full scan ion chromatograms throughout the incubation period. The Rt and fragmentation profiles observed in positive and/or negative ion ESI modes for these peaks were similar to the standards of rebaudioside A, stevioside, rebaudioside B, rubusoside, SG(1), SG(2) and steviol (Table 2).

The proposed scheme for biotransformation of rebaudioside A based on the identification of human intestinal metabolites, is shown in Fig. 5.

3.4. Steviol

Negative ESI/MS full scan and monitor ion scan mass spectra were obtained from steviol anaerobically incubated with pooled human faecal homogenates for 0, 8 and 24 h. No peaks except for steviol appeared on either of the scan mass spectra throughout the incubation period.

3.5. α-Monoglucosylstevioside

Negative ESI/MS full scan and monitor ion scan mass spectra were obtained from α-monoglucosylstevioside anaerobically incubated with pooled human faecal homogenates for 0, 8 and 24 h. Monitor ion peaks at *m/z* 1025 (Rt: 7.6 and 8.4 min), *m/z* 1187 (Rt: 7.8 min), *m/z* 863 (Rt: 9.4 min), *m/z* 701 (Rt: 12.9 min), *m/z* 539 (Rt: 14.1 and 17.8 min) and *m/z* 317 (Rt: 22.1 min) were identified on the monitor ion chromatograms. The Rt and fragmentation patterns observed in positive and/or negative ion ESI modes for these peaks were consistent with the standards of α-monoglucosylstevioside, α-diglucosylstevioside, stevioside, rubusoside, SG(1), SG(2) and steviol (Table 2). However, an unknown peak at *m/z* 1025 (Rt: 6.9 min) appeared at 8 h and thereafter was eliminated at 24 h on the monitor ion chromatograms. The MS/MS fragmentation in positive ion mode for this peak generated a product ion of *m/z* 319 as the aglycone, steviol, suggesting that the peak may be a metabolite originated from α-diglucosylstevioside, a component in α-monoglucosylstevioside. Finally, only the steviol peak was found on the full scan ion chromatogram at a substrate concentration of 0.2 mg/ml after 24 h of incubation.

The proposed scheme for biotransformation of α-monoglucosylstevioside, based on the identification of human intestinal metabolites, is shown in Fig. 4.

3.6. α-Monoglucosylrebaudioside A

Negative ESI/MS full scan and monitor ion scan mass spectra were obtained from α-monoglucosylstevioside anaerobically incubated with pooled human faecal homogenates for 0, 8 and 24 h. Monitor ion peaks at *m/z* 1187 (Rt: 8.1 and 8.3 min), *m/z* 1025 (Rt: 9.2 min), *m/z* 863 (Rt: 8.6 and 9.4 min), *m/z* 701 (Rt: 12.9 min), *m/z* 539 (Rt: 14.1 and 17.8 min) and *m/z* 317 (Rt: 22.1 min) were identified on the monitor ion chromatograms. The Rt and fragmentation patterns observed in positive and/or negative ion ESI modes for these peaks were compatible with the standards of α-monoglucosylrebaudioside A, rebaudioside A, rebaudioside B, stevioside, rubusoside, SG (1), SG (2) and steviol (Table 2). However, unknown peaks at m/z 1187 (Rt: 6.1 and 7.8 min) appeared at 8 h and then disappeared at 24 h on the monitor ion chromatograms. The MS/MS fragmentations in positive ion mode for these peaks generated a common product ion of *m/z* 319 as steviol, the aglycone, suggesting that these peaks may be metabolites originated from impurities existing in α-monoglucosylrebaudioside A. Finally, only the steviol peak was found on the full scan ion chromatogram at the substrate concentration of 0.2 mg/ml after 24 h of incubation.

The proposed scheme for biotransformation of α-monoglucosylrebaudioside A, based on the identification of human intestinal metabolites, is shown in Fig. 5.

3.7. Substrate degradation and conversion to steviol from the substrate

3.7.1. Stevioside

Fig. 6 shows the time-dependency of stevioside degradation (A) and conversion to steviol (B) at substrate

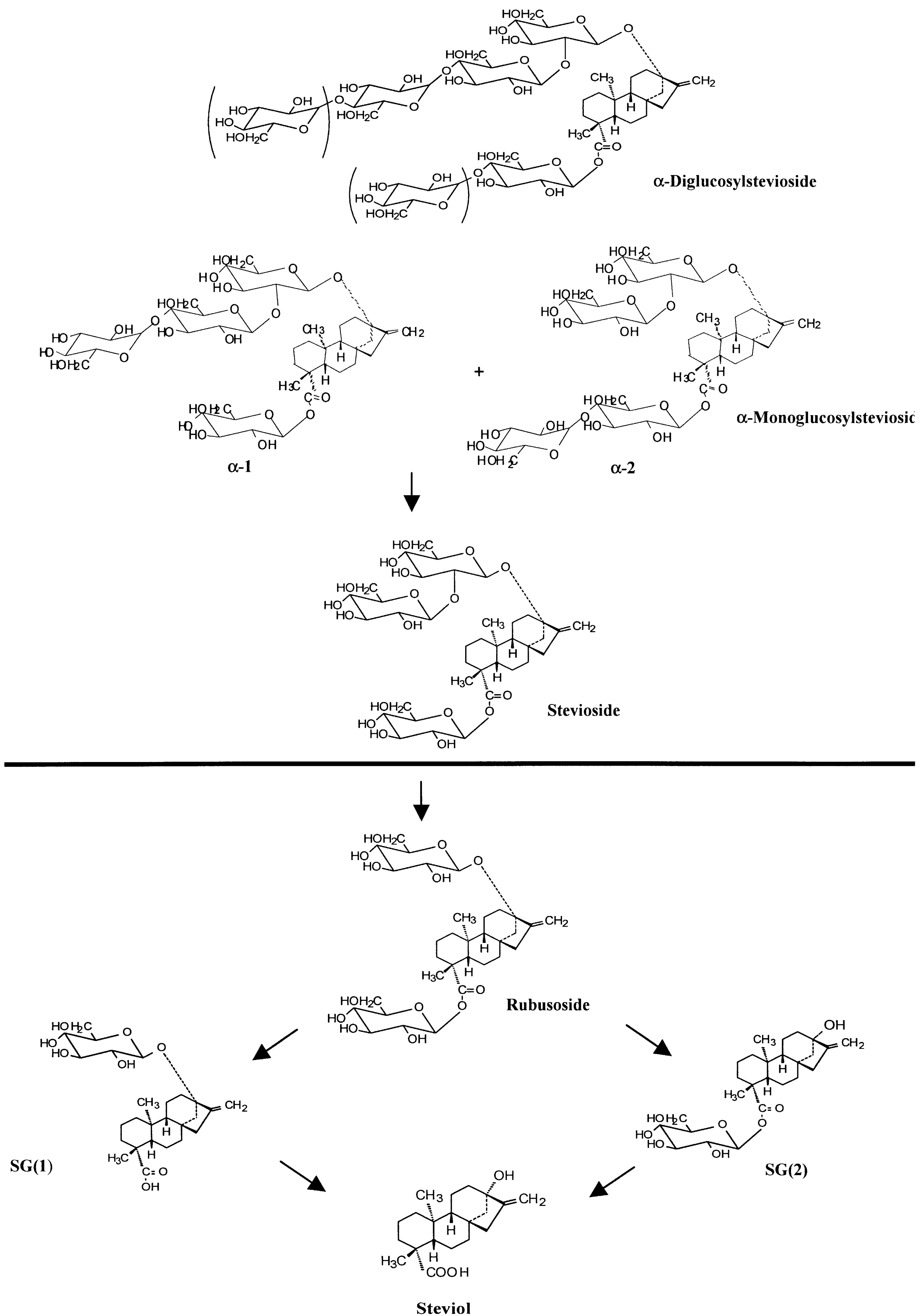

Fig. 4. Proposed metabolic pathways of stevioside and α-monoglucosylstevioside in human intestinal microflora.

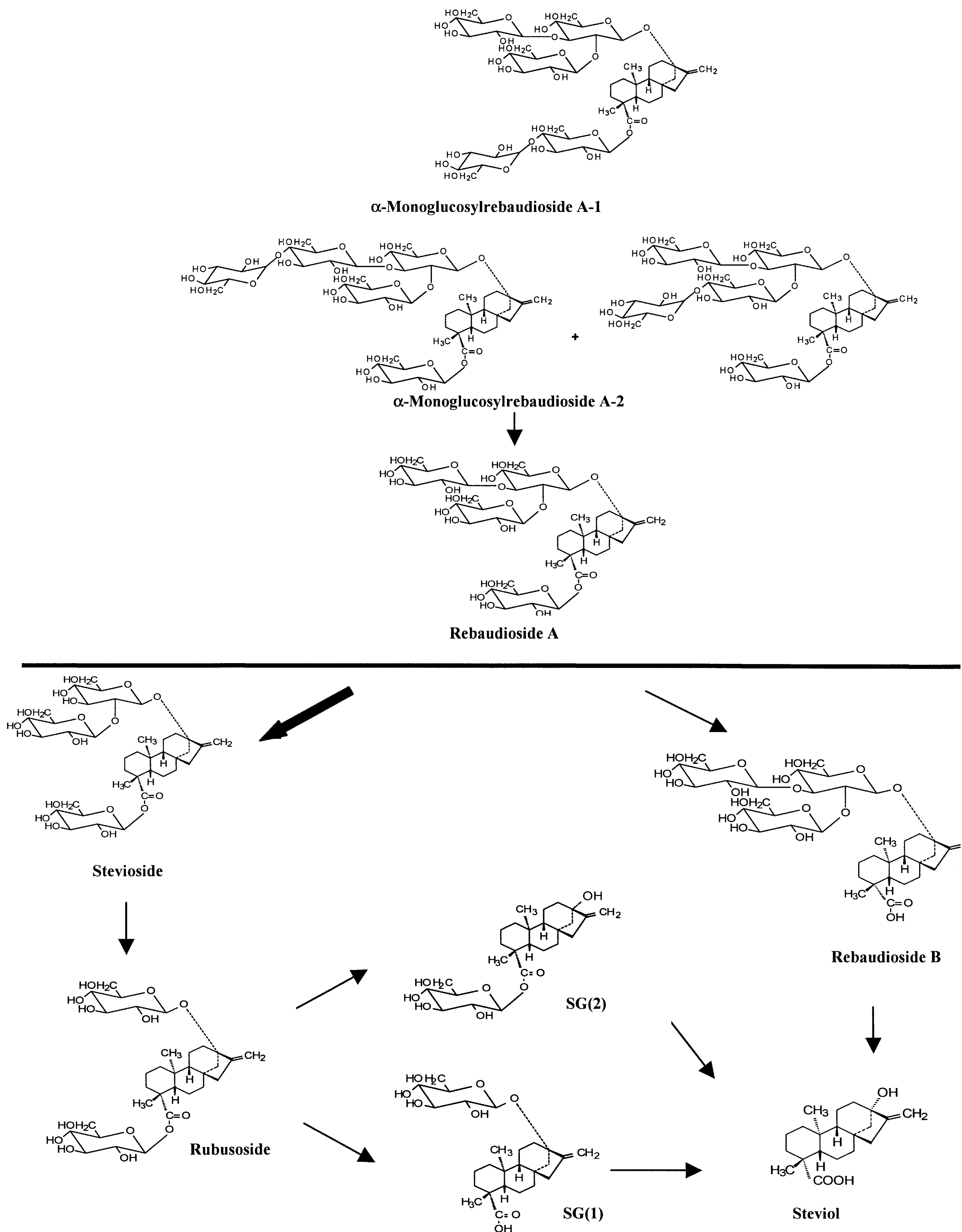

Fig. 5. Proposed metabolic pathways of rebaudioside A and α-monoglucosylstevioside A in human intestinal microflora.

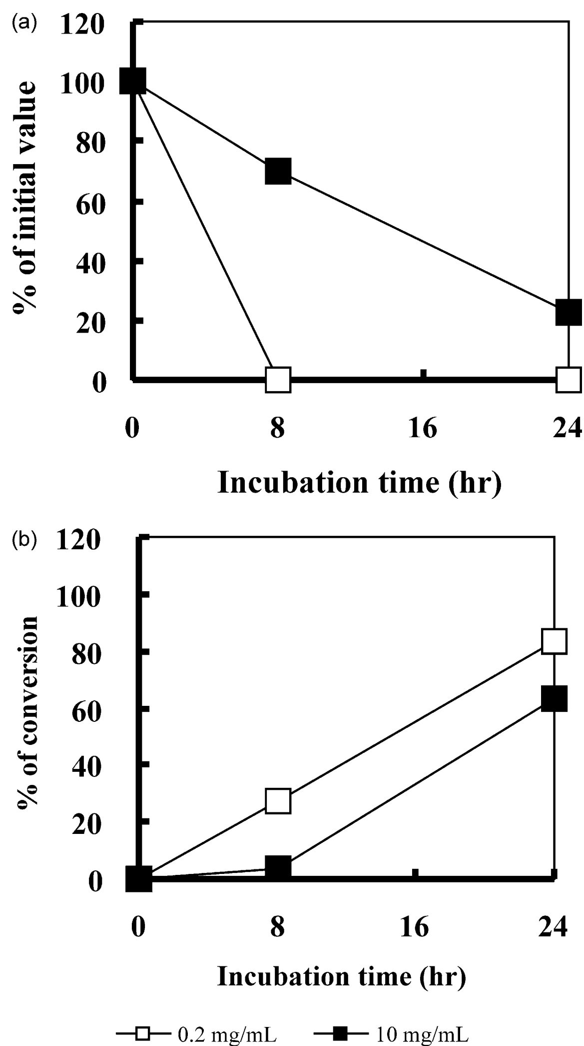

Fig. 6. Time-dependency of stevioside degradation by pooled human faecal homogenates. Stevioside (0.2 or 10 mg/ml) was incubated with pooled human faecal homogenates obtained from five male healthy volunteers (faeces:buffer ratio 1:50, w/v) at 37 °C under anaerobic conditions. Each point represents the mean of duplicate determinations. The values are expressed as mol of steviol equivalent per that of stevioside × 100.

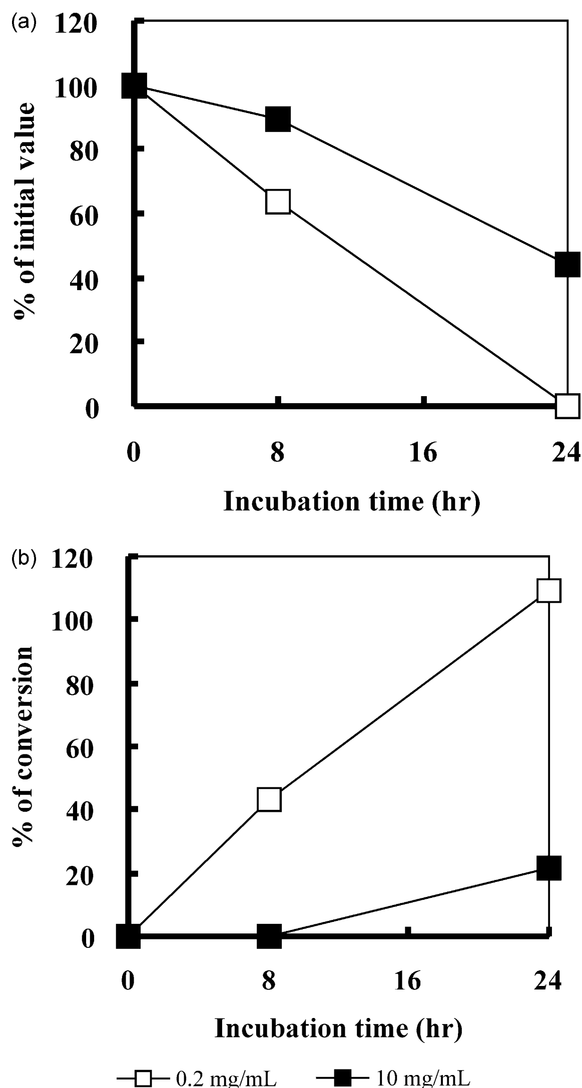

Fig. 7. Time-dependency of rebaudioside A degradation by pooled human faecal homogenates. Rebaudioside A (0.2 or 10 mg/ml) was incubated with pooled human faecal homogenates obtained from five male healthy volunteers (faeces:buffer ratio 1:50, w/v) at 37 °C under anaerobic conditions. Each point represents the mean of duplicate determinations. The values are expressed as mol of steviol equivalent per that of rebaudioside A × 100.

concentrations of 0.2 and 10 mg/ml by pooled human faecal homogenates. Stevioside was degraded in a time-dependent manner throughout the incubation period. After 8 h of incubation, stevioside concentration decreased below the limit of detection (<6 μg/ml) at the low concentration, whereas 23% of the stevioside remained at the high concentration after 24 h of incubation. The conversion rates of stevioside to steviol were 84 and 63% at the low and high substrate concentrations, respectively.

3.7.2. Rebaudioside A

Fig. 7 shows the time-dependency of rebaudioside A degradation (A) and conversion to steviol (B) at substrate concentrations of 0.2 and 10 mg/ml by pooled human faecal homogenates. At the low concentration, rebaudioside A decreased linearly and was completely eliminated during the incubation period (<6 μg/ml). At the high concentration, an apparent decrease in rebaudioside A was observed after 8 h and 44% of it remained after 24 h of incubation. On the other hand, the conversion rates of rebaudioside A to steviol were 109 and 22% at the substrate concentrations of 0.2 and 10 mg/ml, respectively. The higher value at 0.2 mg/ml (109%) was due to the variability of analyzing rebaudioside A. Therefore, rebaudioside A appeared to be completely converted to steviol after 24 h of incubation.

3.7.3. Steviol

We examined the time-dependency of steviol degradation under anaerobic conditions at substrate

concentrations of 0.08 (almost equimolar to stevioside) and 0.2 mg/ml by using pooled human faecal homogenates (Fig. 8). Steviol was almost unchanged at both concentrations throughout the incubation period. Most (99.2 and 96.4%, respectively) of the steviol remained at substrate concentrations of 0.08 and 0.2 mg/ml, after 24 h of incubation.

3.7.4. Stevia mixture

Fig. 9 shows the time-dependency of degradation of stevia mixture components at substrate concentrations of 0.2 and 10 mg/ml by pooled human faecal homogenates. At the low concentration, the relative elimination rates of substrates were stevioside > rebaudioside C > rebaudioside A and all substrates decreased below the limits of detection (<6 μg/ml) after 24 h of incubation. At the high concentration, stevioside decreased linearly and was completely eliminated after 24 h of incubation. Similarly, an apparent decrease in rebaudioside C was seen, 7% remaining after 24 h of incubation. On the other hand, rebaudioside A was almost unchanged, 98% remaining after 24 h of incubation.

The conversion of stevia mixture to steviol increased in a time-dependent manner and steviol levels in the incubation suspension were 0.154 and 4.99 mg/ml at substrate concentrations of 0.2 and 10 mg/ml, respectively after 24 h (figure not shown). Calculating from the total concentrations of main components (stevioside, rebaudioside A, rebaudioside C and dulcoside A), the conversion rates were lower than 85 and 55% at the substrate concentrations of 0.2 and 10 mg/ml, respectively, at 24 h.

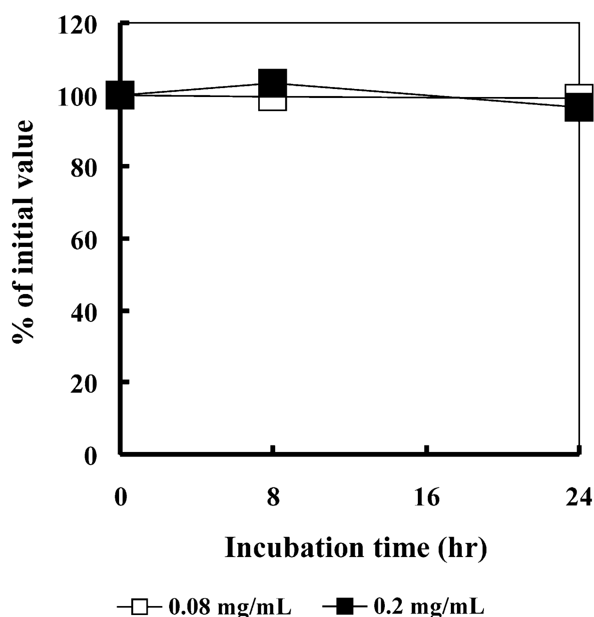

Fig. 8. Time-dependency of steviol degradation by pooled human faecal homogenates. Steviol (0.2 or 10 mg/ml) was incubated with pooled human faecal homogenates obtained from five male healthy volunteers (faeces:buffer ratio 1:50, w/v) at 37 °C under anaerobic conditions. Each point represents the mean of duplicate determinations.

3.8. Enzymatically modified stevia

We studied the time-dependency of degradation of enzymatically modified stevia at substrate concentrations of 0.2 and 10 mg/ml using pooled human faecal homogenates.

The conversion of enzymatically modified stevia to steviol increased in the time-dependent manner and conversion rates were approximately 80 and 17% at substrate concentrations of 0.2 and 10 mg/ml, respectively, at 24 h.

4. Discussion

The pharmacokinetics of stevioside are well documented from in vivo and in vitro studies in rats, but the metabolism of stevioside and its related compounds in humans is basically unknown. To our knowledge, this work provides the first evidence on metabolism of stevia mixture and enzymatically modified stevia by human intestinal microflora. Using the LC/ESI/MS and LC/MS/MS data, we were able to determine the structures of the hydrolysis products. The metabolic pathways of stevioside and α-monoglucosylstevioside as proposed from these data are summarized in Fig. 3. Stevioside is hydrolyzed via rubusoside, SG(1) and SG(2), and (finally) to steviol, the aglycone. The decomposition of α-monoglucosylstevioside is similar to that of stevioside after α-deglucosylation. Fig. 4 shows the proposed metabolic pathways for rebaudioside A and α-monoglucosylrebaudioside A. The predominant pathway suggests that rebaudioside A is hydrolyzed via stevioside, and eventually to steviol, whereas the minor pathway indicates that rebaudioside A is metabolized via rebaudioside B, and finally to steviol. Also, the intestinal metabolism of α-monoglucosylrebaudioside A is similar to that of rebaudioside A after α-deglucosylation. In this study, we have determined whether steviol would be further metabolized in human intestinal microflora. Previous studies (Pezzuto et al., 1985; Compadre et al., 1988; Hutapea et al. 1997) reported that steviol is oxidized to produce hydroxysteviol (mol. wt 334), 15-oxo-steviol (mol. wt 332), steviol-16,17-epoxide (mol. wt 334) or dihydroxysteviol (mol. wt 352) by a variety of pathways. Throughout the incubation period, there was no peak except for steviol on both the full scan ion and monitor ion (*m*/*z* 333, *m*/*z* 331 and *m*/*z* 351) chromatograms of steviol obtained by negative ion LC/ESI/MS analysis (data not shown). No degradation of steviol at high or low concentrations was observed over the 24-h incubation period. Furthermore, there were no other peaks except for steviol on LC/MS chromatograms of stevia mixture and enzymatically modified stevia at the low concentration after 24 h of incubation. Therefore, steviol is confirmed to be a final product from ste-

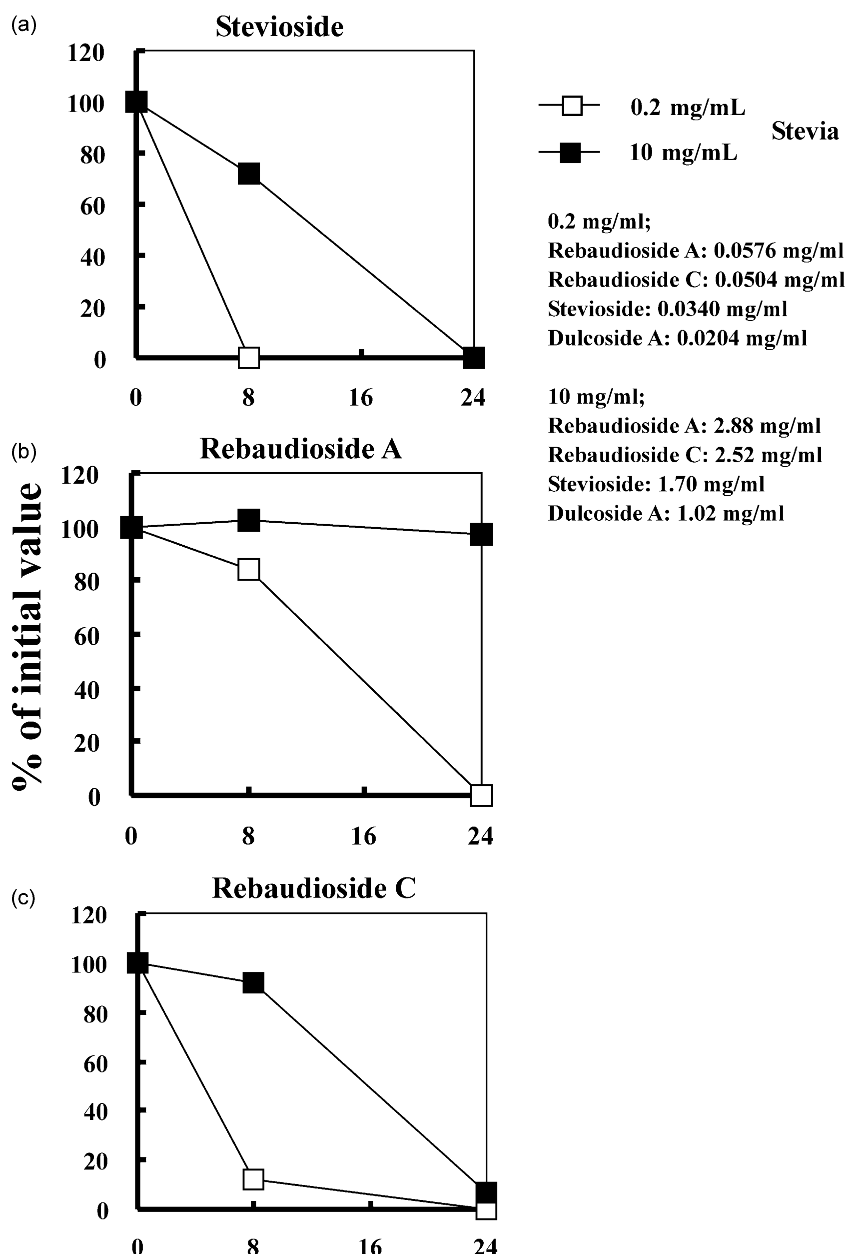

Fig. 9. Time-dependency of Stevia mixture degradation by pooled human faecal homogenates. Stevia mixture (0.08 or 0.2 mg/ml) was incubated with pooled human faecal homogenates obtained from five male healthy volunteers (faeces:buffer ratio 1:50, w/v) at 37 °C under anaerobic conditions. Each point represents the mean of duplicate determinations.

via and its related compounds including enzymatically modified stevia in human intestinal microflora. A recent study (Hutapea et al., 1997) suggests that stevioside was metabolized to 89% of steviol and 13.5% of steviol-16,17- epoxide after 48-h incubation, and thereafter steviol-16,17-epoxide was reduced to steviol by human intestinal microflora after 96 h of incubation under anaerobic conditions. The discrepancy between this study and our study may be due to two factors. First, there is a possibility that the experimental regimen of Hutapea et al. (1997) was not sufficiently anaerobic, allowing the oxidation of the steviol to occur. Secondly, analysis using HPLC-UV (as employed in the earlier study) is not highly specific, so another peak might have been erroneously attributed to steviol-16,17-epoxide.

The metabolism of stevia-related compounds in humans and rats has also been compared. Stevioside (2.5 mg/ml) and rebaudioside A (3 mg/ml), respectively, were completely degraded to steviol by a rat whole cell suspension within 48 and 144 h, whereas steviol (0.2 mg/ml) was stable over 144 h (Wingard et al., 1980). In this study, it was observed that the conversion rate of ste-

vioside to steviol was more rapid than that from rebaudioside A by human faecal homogenates. This suggests that there is apparently no species difference in the intestinal metabolism of stevioside, rebaudioside A and steviol between humans and rats. Quantitative and qualitative similarities between microflora of the rat caecum and that of human lower bowel have been reported in the literature (Lucky, 1972).

Stevia mixture, but neither stevioside nor rebaudioside A alone, is used as a food additive on the market. The in vitro metabolism of stevia mixture in human intestinal microflora is slightly different from that of the substrate alone because of substrate–substrate interaction. The degree of elimination of rebaudioside A at a concentration of 10 mg/ml was 55.7%, whereas that of stevia mixture (including 2.88 mg/ml rebaudioside A) at 10 mg/ml was only 2.5% after 24 h. The degradation of rebaudioside A by human faecal homogenates tended to be slower in the presence of other components (stevioside, rebaudioside C) than in their absence. This result suggests the possibility that the same microbial cell(s) or same enzyme(s) may contribute to the hydrolysis of components or metabolites of stevia mixture, competing among these compounds for the same enzyme(s). For rebaudioside A, having a complex structure, it may be more difficult to access the affinity site of enzyme(s) compared to other components or their metabolites (including stevioside).

In the presence of both rebaudioside A and rebaudioside C, in stevia mixture, it is observed that the elimination of rebaudioside C was more rapid than that of rebaudioside A. Rebaudioside A has $\beta(1>2)$-linked glucose on the 13-position of the site, whereas rebaudioside C has $\alpha(1>2)$-linked rhamnose on the same site. This suggests that the metabolism is stereoselective, with the human intestinal microflora ‘‘preferring’’ the latter chemical structure.

In summary, stevia-related compounds (including its α-derivative, enzymatically modified stevia) are degraded finally to steviol, the aglycone by human faecal homogenates. There is no apparent species differences in the intestinal metabolism of stevia-related compounds between humans and rats. Substratesubstrate interaction is observed in the in vitro metabolism of stevia mixture in human intestinal microflora, suggesting that this phenomenon may occur in vivo. Further studies need to be performed to investigate the absorption and hepatic metabolism of stevia-related compounds in human.

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