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Isolation and physiological activities of a new amino acid derivative from Korean red ginseng

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Abstract

Three unknown ninhydrin positive substances (UK-I, UK-II and UK-III) were detected with an amino acid analyzer in a water extract of Korean red ginseng. One of them (UK-II) was isolated and determined to be maltulosyl arginine (Arg-Fru-Glc) on the basis of chemical and spectroscopic evidence. Another one (UK-III) was identified as Arg-Fru. Maltulosyl arginine, but not Arg-Fru, is a newly identified amino acid derivative. The Korean red ginseng was shown to contain more amounts of maltulosyl arginine than the white ginseng. Maltulosyl arginine was found to be produced by the Maillard reaction of maltose with arginine during the heating process involved in preparation of the red ginseng. Maltulosyl arginine was found to inhibit maltase activity. Based on these results, the physiological significance of this new compound is discussed.

Key words red ginseng, maltulosyl arginine, Arg-Fru-Glc, Maillard reaction.

Abbreviations Arg-Fru, Arginyl-fructose ; Arg-Fru-Glc, Arginyl-fructosyl-glucose ; ^{13}C-NMR, carbon 13 nuclear magnetic resonance ; DEPT, distortionless enhancement by polarization transfer ; EI, electron impact ; GC-MS, gas chromatograph mass spectrometer ; ^{1}H-NMR, proton nuclear magnetic resonance ; HPLC, high performance liquid chromatography ; TFA, trifluoroacetic acid ; TLC, thin layer chromatography ; TMS, tetramethylsilane ; TOF-MS, time of flight mass spectrometer.

Introduction

Korean red ginseng is a medicinal plant long used in the treatment of various pathological states including general complaints such as headache, shoulder-ache, chills, anorexia and diabetes. During the last twenty years, there have been extensive physiological and biochemical studies on the mechanism of its effects in animals and humans. Most of these studies have been conducted on saponin fractions of ginseng.[1-4] However, we have isolated various physiologically active substances from a non-saponin fraction of Korean red ginseng[5-8]: namely, adenosine, pyroglutamic acid, acidic polysaccharide and dencichine. This paper reports the isolation of a new nitrogenous compound identified as maltulosyl arginine, from a non-saponin fraction of Korean red ginseng, the mechanism of its production and its physiological actions.

Materials and Methods

Korean ginseng : Red ginseng powder (*Panax ginseng* C. A. MEYER.) was kindly provided by Nikkan Korai Ninjin Co. Ltd., Kobe, Japan and Korea Ginseng and Tobacco Research Institute, Deajeon, Korea. White ginseng preparations obtained after cultivation of plants for 1, 2, 3, 4, 5 and 6 years were kindly supplied by Dr. Hoon Park of the Korean Ginseng and Tobacco Research Institute.

Other materials : ^{14}C (U)-maltose was purchased from Amersham Japan (Tokyo, Japan) and α-glucosidase isolated from *Saccharomyces* sp., was

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Table I Program of amino acid analysis with Hitachi analyzer.

Buffer*	PF-1	PF-2	PF-3	PF-4	PF-5	PF-6	PF-1
Time (min)	0-80	80-128	128-165	165-188	188-220	220-230	230-270

Column temp. (°C)	36	44	50	64	68	36
Time (min)	0-32	32-50	50-125	125-215	215-235	235-270

Column : 2619 F (4×150 mm), flow rate : 0.325 ml/min.

*Composition of buffer (per 1 L)

Item	PF-1	PF-2	PF-3	PF-4	PF-5	PF-6
Litium citrate ($4H_2O$)	9.80g	9.80g	9.80g	9.80g	47.00g	-
LiCl	2.12g	6.36g	29.67g	38.15g	29.67g	-
Citric acid (H_2O)	34.00g	12.00g	12.00g	3.30g	-	-
LiOH	-	-	-	-	-	8.40g
Ethanol	40ml	30ml	-	-	-	-
Thiodiglycol	5ml	5ml	-	-	-	-
BRIJ-35 (25 g/100 ml)	4ml	4ml	4ml	4ml	4ml	4ml
Caprylic acid	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
Li concentration (N)	0.155	0.255	0.805	1.00	1,20	0.200
pH	3.0	3.7	3.3	4.1	7.0	-

obtained from Funakoshi Co.(Tokyo, Japan).

Amino acid analysis : A water extract of Korean red ginseng was filtered through a 0.2 μm membrane filter and the filtrate was subjected to amino acid analysis (Hitachi type 835 apparatus). A Hitachi 2619F column was used and the analysis was carried out according to the program shown in Table I. Amino acids and related compounds were detected by the ninhydrin reaction.

Isolation of a new nitrogenous compound (UK-II) : Korean red ginseng powder was mixed with 10 volumes of water with stirring for 12 h at 4°C. Then, the mixture was centrifuged and the resultant supernatant was dialyzed against water for 12 h at 4°C, using a dialysis membarne (Seamless Cellulose Tubing 36/32, VISKASE SALES Co.). The outer dialysate was centrifuged and the supernatant was again dialyzed in the same way. The outer dialysates thus obtained were combined and freeze-dried. The resulting powder was dissolved in water at a concentration of 20 mg/ml, filtered through a 0.2 μm filter membrane and subjected to amino acid analysis. A new nitrogenous compound (UK-II) was collected with a fraction collector, and desalted by reverse phase HPLC, using a TSKgel ODS 120-T column (4.6 mm I.D.× 25 cm, TOSOH, Tokyo) and 0.1 % TFA as eluent.

Chemical analysis : The carbohydorate moiety of the new nitrogenous compound (UK-II) was examined by GC-MS analysis after trimethylsilylation of α-glucosidase- and alkali-treated UK-II. One ml of TMS reagent (TMS-HT, Tokyo-Kasei Co., Tokyo) was added to 10 mg of α-glucosidase- or a alkali-treated UK-II and stirred for 30 sec. Then the reaction mixture was let to stand for 5 min and centrifuged for 10 min at 1,500×g. The supernatant was analyzed with GC-MS (EI, at 70eV), using a Shimadzu QP-1000 mass-spectrometer. The analytical conditions of gas chromatograph were as follows : column packing, 3 % OV-17 gas chrom Q, 80~200 mesh (GL Sciencies Co., Tokyo) ; glass column, 3.2 mm×2 m ; column temperature 160~200°C (1°C/min) ; injection temperature, 210°C ; and flow rate of He gas, 40 ml/min. The molecular weight of UK-II was determined with a matrixassisted laser desorption ionization TOF-MS (Shimadzu Kratos Kompact MALDI III), using gentisic acid as a matrix. ^{1}H-and ^{13}C-NMR spectra were recorded at 270 and 67.8 MHz, respectively, with a JEOL GSX-270 spectrometer in D_2O at 25°C. Chemical shifts were expressed in δ ppm relative

to internal HDO (δ 4.70) in ^{1}H-NMR and external dioxane (δ 67.40) in ^{13}C-NMR. Multiplicity in the ^{13}C-NMR spectrum was determined with a DEPT program.

Chemical synthesis of maltulosyl arginine : Maltose-monohydrate (900 mg) and L-arginine (220 mg) were dissolved in 5 ml of glacial acetic acid, stirred for 1 h at 75~80°C and then concentrated to dryness. The dried sample (300 mg) was applied to 6 pieces of TLC plate (silica gel 60 F_{254} 20×20 cm, layer thickness 0.5 mm, Merck Co.) and developed with butanol-acetic acid water (2 : 1 : 1, V/V/V). The ninhydrin-positive spot at Rf 0.2 was scraped off and extracted with water. The extract was concentrated and applied to a cation exchange column (DOWEX 50W-×2, Dow Chemical Co.) to remove silica gel. After washing with water, maltulosyl arginine was eluted from the column with 1N-NH_4OH and analysed with ^{1}H-NMR. Amino acid analysis of the reaction mixture showed that 80.8 % of arginine had been converted to *maltulosyl arginine*.

Assay of maltase activity : Mucosal scrapings from the jejunum of male Wistar-King strain rats were homogenized in 80 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 2,000×g for 10 min and the resulting supernatant was used as an enzyme solution. The reaction mixture for assay of maltase activity consisted of 80 μl of 5 mM ^{14}C-maltase (1.6 μ Ci), 10 μl of sample solution and 10 μl of enzyme solution. After incubation for 30 min at 37°C, the reaction was stopped by heating at 100°C for 2 min. A sample of 5 μl of the supernatant of the reaction mixture was spotted onto TLC plates and developed with acetone-water (9 : 1, V/V). Maltase activity was expressed as the amount of glucose liberated in μ mol/min/mg protein.

Analysis of data : Statistical analyses were done by analysis of variance (ANOVA).

Results

The outer dialysate of the water extract from Korean red ginseng was examined with the amino acid analyzer. As shown in Table II, the main free amino acids in this fraction were arginine and asparagine, β-alanine, asparatic acid and alanine, other free amino acids being present at a lower concentration. In addition to these amino acids, the outer dialysate contained three unknown ninhydrin-positive substances, UK-I, UK-II and UK-III which were eluted after glutamine, before β-alanine and after γ-amino-butyric acid respectively (Fig. 1). Of these unknown substances, the content of UK-II was the highest judging from its area ratio shown in Table II. Therefore, we tried to identify UK-II.

Table II Free amino acid contents in water extract of Korean red ginseng.

Abbreviation	Amino acid	mg/g	Area ratio
ASP	Aspartic acid	1.41	0.08
THR	Threonine	0.48	0.03
SER	Serine	0.41	0.03
GLU	Glutamic acid	0.24	0.01
$GLUNH_2$	Glutamine	0.10	0.00
UK-I	Unknown-I	-	0.02
GLY	Glycine	0.04	0.00
ALA	Alanine	1.20	0.08
VAL	Valine	0.21	0.01
CYS	Cystine	0.45	0.01
ILE	Isoleucine	0.27	0.01
LEU	Leucine	0.31	0.02
TYR	Tyrosine	0.43	0.02
PHE	Phenylalanine	0.20	0.00
UK-II	Unknown-II	-	0.34
β-ALA	β-Alanine	3.60	0.05
γ-ABA	γ-Amino-n-butyric acid	0.81	0.03
UK-III	Unknown-III	-	0.09
LYS	Lysine	0.22	0.01
HIS	Histidine	0.32	0.01
ARG	Arginine	21.00	1.00
$ASPNH_2$	Asparagine	4.33	0.03

UK-II isolated with the amino acid analyzer was found to liberate arginine, fructose and glucose after treatment with 3N NH_4OH at 100°C for 1 h. The arginine liberated was identified with the amino acid analyzer. And the carbohydrate moieties were identified by GC-MS after trimethylsilylation. The retention times of those trimethylsilylated carbohydrates (10.85, 11.40, 16.25 and 21.25 min) were identical with those of authentic trimethylsilylated fructose (10.70 and 11.25 min) and glucose (15.50 and 20.25 min). Analysis on the ^{13}C-NMR spectrum suggested the presence of two saccharide moieties in addition to one arginine moiety (Table III). Thus UK-II might be

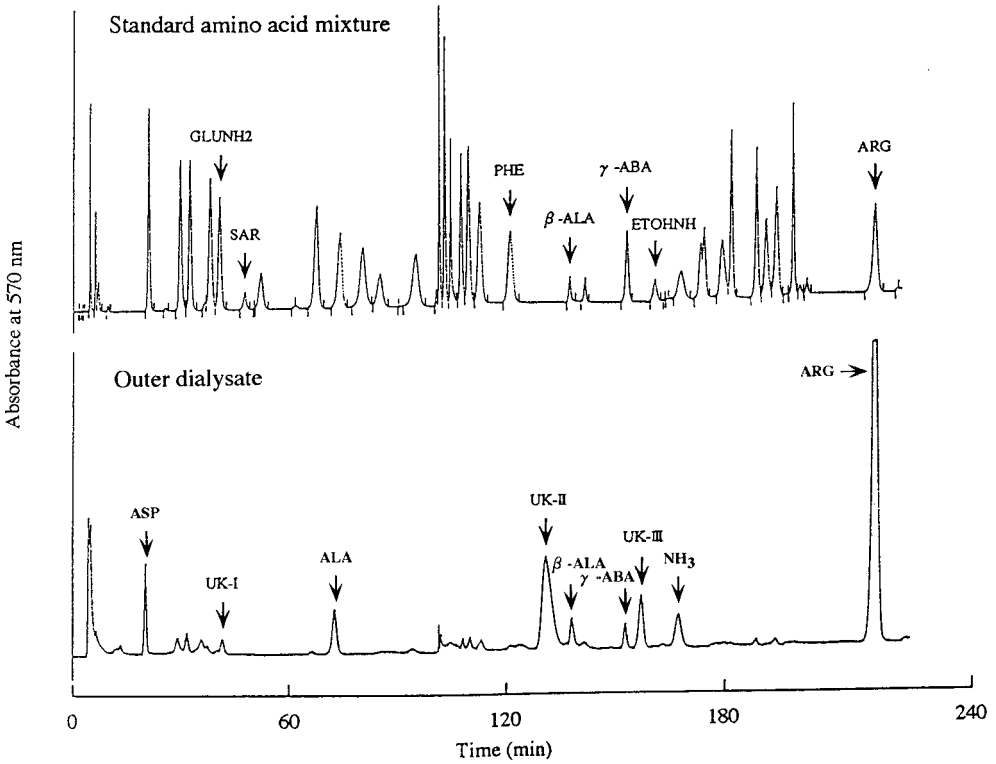

Fig. 1 Analysis of amino acids in the outer dialysate of the water extract from Korean red ginseng.

Table III ^{13}C-NMR chemical shifts of predominant form[a] of UK-II in D_2O.

Carbon[b]	δ	Multiplicity
1	173.50	s
2	63.13	d
3	27.26	t
4	24.71	t
5	41.23	t
6	157.57	s
1'	53.14	t
2'	96.19	s
3'	69.72	d
4'	78.38	d
5'	70.02	d
6'	64.76	t
1"	101.38	d
2"	72.49	d
3"	73.55	d
4"	70.34	d
5"	73.19	d
6"	61.30	t

a) Fructose moiety is β-pyranose form.
b) Assignment of disaccharide moiety was carried out by comparison with the data of maltulose.[11]

composed of arginine, fructose and glucose with the ratio 1 : 1 : 1, After incubation of 0.2 mg of UK-II with 0.1 U of α-glucosidase in 40 μl of 10 mM phosphate buffer (pH 6.8) containing 15 mM EDTA at 37°C for 1 h, the liberation of glucose was demonstrated by GC-MS after trimethylsilylation (retention time : 16.25 and 21.40 min). This result suggested that glucose residue did not link to arginine residue, but fructose moiety. Thus the sequence of arginine, fructose and glucose in UK-II might be Arg-Fru-Glc, not Arg-Glc-Fru and Fru-Arg-Glc. The molecular weight of UK-II was found to be 498 by the matrix-assisted laser desorption ionization TOF-MS (m/z 498.4), which is consistent with its proposed chemical structure ($C_{18}H_{34}N_4O_{12}$=498).

It was reported that free fructose is present as 4 isomers which are α-pyranose, β-pyranose, α-furanose and β-furanose forms in the water solution and α-pyranose form is negligible in comparison with other three forms.[9,10] Analysis of the ^1H-NMR spectrum of UK-II showed that the proton at C-1 position

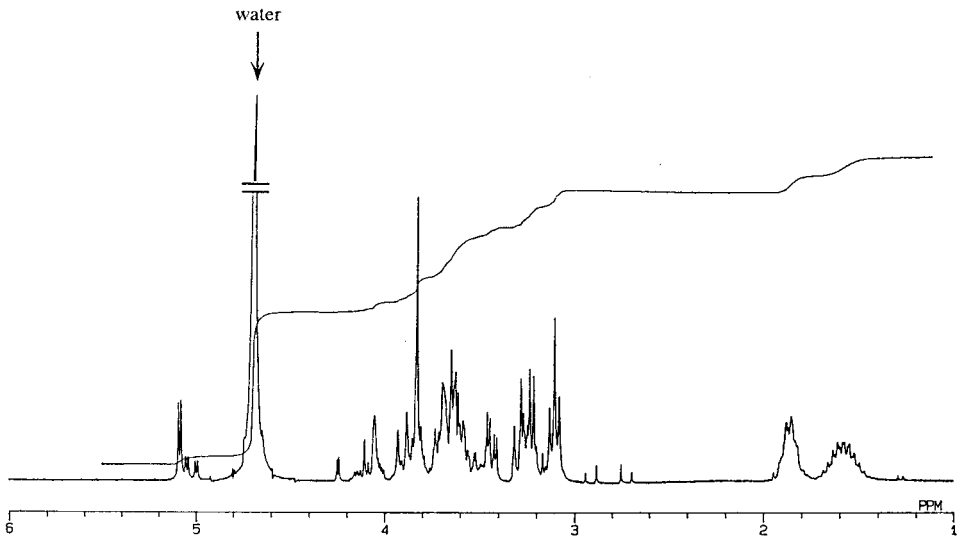

Fig. 2 ^{1}H-NMR spectra of UK-II at 25°C in D_2O.

of glucose residue split into 3 peaks, δ 5.00 (d, J=3.66 Hz), 5.05 (d, J=3.66 Hz) and 5.09 (d, J=3.66 Hz), suggesting the presence of isomers which might be α-furanose, β-furanose and β-pyranose due to the comformational change of fructose moiety (Fig. 2). The ^{13}C-NMR spectrum showed also a complicated pattern as the ^{1}H-NMR spectrum. These results indicated that the glucose and arginine residue did not associate with the C-2, C-5 and C-6 of fructose residue. If the C-5 or C-6 position is substituted, fructose residue can not be interchanged from pyranose to furanose form, and vice versa. Furtermore, if the C-2 position is substituted, anomeric configuration of fructose residue is fixed. Analysis of the ^{13}C-NMR spectrum showed upfield shift of C-1 position of fructose residue and α-CH of arginine, suggesting substitusion of C-1 hydroxyl group of fructose residue by amino group of arginine residue. Analysis of the ^{1}H-NMR spectrum showed a small J value (3.66 Hz) of an anomeric proton of the glucose moiety, indicating an α-configuration. All these findings suggested that glucose was associated with fructose by α 1-3 or α 1-4 bonding.

Although Korean red ginseng contained a large amount of UK-II (5.37 %), only a trace of UK-II was found in Korean white ginseng, as shown in Table IV. After cultivation for 1, 2 and 3 years the white ginseng contained no detectable UK-II, and its UK-II contents after cultivation for 4, 5 and 6 years were only 0.09, 0.08 and 0.20 %, respectively. These results indicated that UK-II was not reduced appreciably during cultivation, but was formed during preparation of the red ginseng. Red ginseng is prepared by heating the ginseng root at 100°C, whereas white ginseng is prepared by sun-drying. Therefore, it seemed likely that UK-II was formed during the heating process involved in preparation of the red ginseng. The results in Fig. 3 confirm this possibility. When a sample of white ginseng (6 years cultivation) was heated with 1 ml of water at 100°C for 3 h, its UK-II content increased from 0.2 % to 0.59 %. Furthermore, UK-II was found to be formed by the Maillard reaction of maltose with arginine in acidic conditions (Fig. 3). UK-III was also

Table IV UK-II content of various ginseng preparations.

Preparations		UK-II content (%)
Red ginseng		4.28
White ginseng (years of cultivation)	(1)	N.D.[a]
	(2)	N.D.
	(3)	N.D.
	(4)	trace
	(5)	trace
	(6)	trace

a) N.D. : not detected.

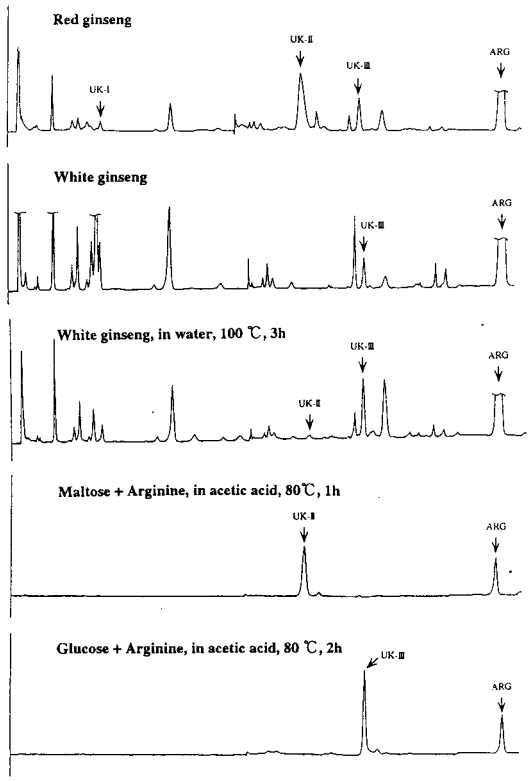

Fig. 3 Amino acid patterns of various preparations. The procedures are described in "Materials and methods" and in the text.

produced by the Maillard reaction. This was demonstrated by heating a mixture of glucose (900 mg) and L-arginine (440 mg) in 5 ml of glacial acetic acid for 2 h at 75~80°C with stirring, concentrating the mixture to dryness and then examining it with the amino acid analyzer. As shown in Fig. 3, the dried sample was found to contain UK-III and arginine as ninhydrin-positive substances, indicating that UK-III was arginyl-fructose (Arg-Fru). Therefore, it may be concluded that UK-II and UK-III are formed by the Maillard reactions of arginine with carbohydrates during heating of Korean ginseng root. The ^{1}H- and ^{13}C-NMR spectra of UK-II were identical with those of maltulosyl arginine (Arg - Fru - Glc) synthesized from maltose and arginine by the Maillard reaction as shown in Fig. 4. The chemical structure of UK-II determined on the basis of all these results is presented in Fig. 5. UK-II is maltulosyl arginine in which the α-1 position of glucose is bound to the α-4 position of fructose and the α-amino group of arginine is bound to C-1 of fructose. Maltulosyl arginine is an equilibrated mixture of three isomers due to the comformation change of its fructose moiety as reported maltulose.[11] The ratio of β-pyranose (β-p), α-furanose (α-f) and β-furanose (β-f) forms of fructose

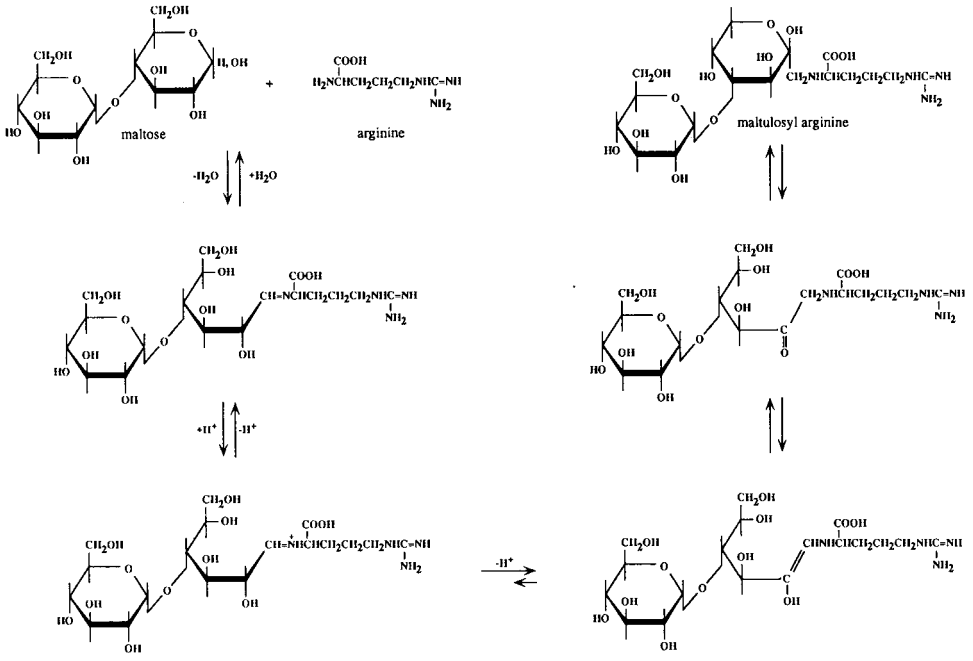

Fig. 4 Maillard reaction of maltose with arginine.

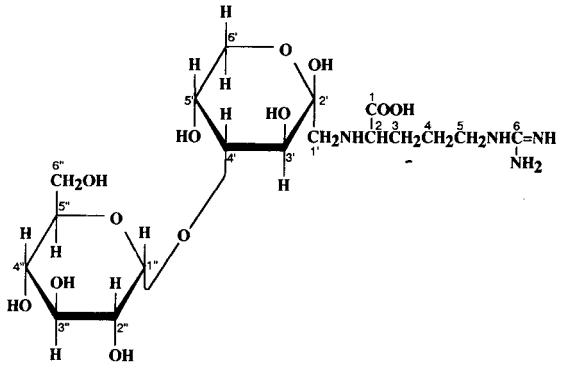

Fig. 5 Chemical structure of UK-II as predominant form.

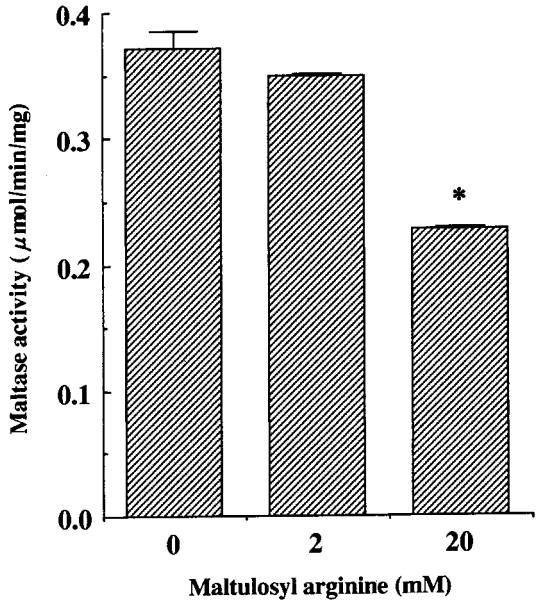

Fig. 6 Effects of maltulosyl arginine on maltase activity. Values are means±S.E. of three separate assays. $*p<0.01$ (vs 0 and 2 mM, ANOVA Scheffe F-test)

moiety is approximately 4 : 1 : 1 calculated by the intensity of C-1 proton signal (β-p, δ 5.09 ; α-f, δ 5.00 ; β-f, δ 5.05) of glucose moiety in ^{1}H-NMR. Thus the β-p form of maltulosyl arginine is shown as a predomonant chemical structure of UK-II in Fig. 5.

Finally, we examined the physiological actions of maltulosyl arginine. We found that maltulosyl arginine at a concentration of 20 mM inhibited maltase activity, as shown in Fig. 6.

Discussion

Maltulosyl arginine isolated from the Korean red ginseng in the present study has not been reported previously. Maltulosyl arginine is easily hydrolyzed in mild conditions such as heating in 3 N NH_4OH at 100 °C for 1 h. Amino acid analysis of natural products is usually done after acid hydrolysis in conditions such as by heating in 6 N HCl at 110°C for 24 h, in which maltulosyl arginine would be completely decomposed. Furthermore, α-amino group of arginine residue in maltulosyl arginine is blocked by maltulosyl group. Thus the ninhydrine reactivity of maltulosyl arginine was extremely reduced. In fact, the content of maltulosyl arginine (5.37 %) is higher than arginin (2.10 %) in water extract of Korean red ginseng, but area ratio of maltulosyl arginine is lower than that of arginine on the amino acid analyzer chromatogram (Fig. 1 and Table II). These facts may be the reason why this compound has not been detected previously in Korean red ginseng.

Although a large amount of maltulosyl arginine (5.37 %) was found in the red ginseng, the white ginseng contained only a trace amount (Table IV). The red ginseng is prepared by heating the ginseng root at 100°C, while the white ginseng is prepared by sun-drying. Therefore, it seems likely that maltulosyl arginine may be formed during the heating process involved in preparation of the red ginseng. In fact, we found that maltulosyl arginine was formed on heating the white ginseng (Fig. 3). Furthermore, maltulosyl arginine was shown to be synthesized by the Maillard reaction of maltose with arginine. Details of the Maillard reaction are shown in Fig. 4. The initial reaction between the aldehyde group of maltose and α-amino group of arginine results in the formation of an aldosylamine. Then an Amadori rearrangement[12] causes the formation of maltulosyl arginine. Therefore, we conclude that maltulosyl arginine is formed by the Maillard reaction during the heating process involved in preparation of Korean red sinseng. We found that Arg-Fru was also formed by the Maillard reaction during the heating process involved in preparation of the red ginseng (Fig. 3). Gordon has reported the synthesis and metabolism of Arg-Fru.[13] Park *et*

al. first reported that arginine constitutes 58 % of the total free amino acid content in Korean red ginseng.[14] Maltose and glucose may be produced by digestion of starch with amylase and maltase in the ginseng root.

Maltulosyl arginine was found to inhibit maltase activity in the mucous layer of rat jejunum (Fig. 6). The inhibitory effect of maltulosyl arginine on maltase may slow down the intestinal absorption of maltose and prevent rapid increase in blood glucose and possibly insulin also. Rapid increases in blood glucose and insulin are known to accelerate lipogenesis in various tissues such as the liver and adipocytes and cause fatty liver, hyperlipaemia and obesity. Therefore, maltulosyl arginine in Korean red ginseng may prevent the development of these disorders and improve these pathological states.

Experiments are now in progress to prove this hypothesis and clarify after physiological actions of this compound.

和文抄録

我々は，韓国産紅参の水抽出液中に，3種類の未知のニンヒドリン陽性物質(UK-I, UK-II, UK-III)が含まれていることを，アミノ酸分析計により発見した。これらの未知物質の一つであるUK-IIについて，単離すると共に化学分析によりその構造がmaltulosyl arginine (Arg-Fru-Glc)であることを明らかにした。更に，UK-IIIの構造についても分析の結果Arg-Fruであることを明らかにした。またmaltulosyl arginineは，新規アミノ酸誘導体であった。このmaltulosyl arginineは，白参よりも紅参中に多量に含まれていた。また，この新規アミノ酸誘導体（maltulosyl arginine）は，紅参の製造過程における加熱によって，マルトースとアルギニンの間のメイラード反応によって合成されることも明らかにした。次に，このmaltulosyl arginineの生理作用について検討を行った結果，この物質は，マルターゼ活性を阻害する事が明らかになった。このような結果は，この新規アミノ酸誘導体が，生理的に重要な働きをする可能性を示唆するものである。

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