Isolation and identification of antioxidants and tyrosinase inhibitors from *Stichopus japonicus*

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Abstract

Sea cucumber has long been used not only as an outstanding tonic food in the Indo-Pacific region but also as a traditional medicine in East Asia. Four compounds, fatty acids, and salts were isolated from *Stichopus japonicus* and identified as adenosine (1), ethyl-α-D-glucopyranoside (2), L-tyrosine (3), 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4), fatty acids, and salts. Adenosine (1), ethyl-α-D-glucopyranoside (2), L-tyrosine (3), and 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4) are responsible for antioxidants. Adenosine (1) and ethyl-α-D-glucopyranoside (2) are also responsible for tyrosinase inhibitors. On the other hand, fatty acids and salts are only responsible for tyrosinase inhibitors. Ethyl-α-D-glucopyranoside (2) and 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4) are first reported in sea cucumber. Furthermore, 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4) was identified as a new compound.

Keywords: Tyrosinase inhibitors, antioxidants, *Stichopus japonicus*, sea cucumber.

Introduction

Sea cucumber, a cylindrical marine invertebrate, has long been used not only as an outstanding tonic food in the Indo-Pacific region but also as a traditional medicine in East Asia due to its multiple biological properties such as antifungal, antivirus, antitumor, anticoagulant, anti-angiogenic, neuritogenic, and immunomodulatory activities (Kitagawa et al., 1976; Mourão et al., 1996; Maier et al., 2001; Kaneko et al., 2003; Tian et al., 2005; Aminin et al., 2006; Mamelona et al., 2007). Triterpene glycosides, commonly known as sea cucumber saponins, are known to be primarily responsible for those properties. Other important active components of sea cucumber include chondroitin sulfate, eicosapentaenoic acid, and ganglioside (Mourão et al., 1996; Kaneko et al., 2003; Zhong et al., 2007).

In spite of many studies on the biological properties, only a few reports are available on the antioxidant property of sea cucumber. Recently, *Cucumaria frondosa*, a common sea cucumber species in coastal waters of the North Atlantic Ocean, and Far-Eastern sea cucumber *Stichopus japonicus* have been demonstrated to have antioxidant activity (Zhong et al., 2007; Mamelona et al., 2007; Husni et al., 2009). Phenolic compounds were suggested to be mainly responsible for the antioxidant activity (Mamelona et al., 2007; Husni et al., 2009), however, key antioxidant substances have not been identified. On the other hand, a pilot study showed that *S. japonicus* had tyrosinase inhibitory activities, however, its active components have also not been identified. To the best of our knowledge, there is no published report on the identification of antioxidants and tyrosinase inhibitors from sea cucumber. Therefore, the objective of this study was to extract, purify, and identify antioxidants and tyrosinase inhibitors from *S. japonicus* body wall.

Materials and methods

Materials

Live specimens of sea cucumber *Stichopus japonicus* were purchased from a fishery market. Mushroom tyrosinase (5370 units/mg), L-3,4-dihydroxyphenylalanine (L-DOPA), L-ascorbic acid, 5-hydroxy-2-hydroxymethyl-4H-4-pyranone (kojic acid), and 2,2´-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) diammonium salt were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Ethanol, methanol, *n*-hexane, dichloromethane, ethylacetate, and *n*-butanol were obtained from Fisher Scientific (Ottawa, ON, Canada). Sephadex LH-20 was purchased from GE Healthcare Bio-Sciences AB (Björkgatan, Uppsala, Sweden), TLC plates (25 DC-platen Kieselgel 60 F_{254}) were obtained from Merck (Frankfurter, Darmstadt, Germany). All the chemicals used in the study were of either analytical grade or HPLC grade.

Extraction and purification

The visceral organs and body fluid of fresh sea cucumber specimens were taken out and the body wall was washed with tap water. The body wall was cut into small pieces and then was extracted over night with 95% ethanol (3 x 2.5 L) at room temperature. The extracts were combined and concentrated in vacuo at 40^{o}C. The concentrated extract was suspended in water (1.3 L); the resultant suspension was partitioned with *n*-hexane (3 x 1.25 L) to give an *n*-hexane fraction (19.53 g). Next, the aqueous layer was partitioned with dichloromethane (3 x 1.25 L) to give a dichloromethane fraction (2.4 g). The aqueous layer was then partitioned successively with ethyl acetate (3 x 1.250 L) and *n*-butanol (3 x 1.25 L) to give 0.13 g of ethyl acetate fraction and 5.79 g of *n*-butanol fraction, respectively, and 131.99 g of water fraction. Then the fifth extracts were dissolved in DMSO and subjected to antioxidant and mushroom tyrosinase inhibition assays.

The butanol fraction was fractionated by Sephadex LH-20 (Ø 0.25 x 38 cm) using methanol as eluent to get two fractions (SJEBa and SJEBb) and compound 1. SJEBb was

fractionated into 4 fractions (SJEBba - SJEBbd) by the same Sephadex using ethanol as eluent and SJEBbc was subjected to Sephadex LH-20 using methanol as eluent to give SJEBbcb and then was purified by repeated HPLC using preparative C_{18} reverse-phase column (Hydrosphere, 5 µm, YMC 250 x 20 mm) with gradient elution using water (A) and methanol (B) consisted of isocratic elution for 20 min with 5% B and linear gradient for 40 min with 5 to 98% B, and isocratic elution for 60 min with 98% B to obtained compound 2 and compound 3. The dichloromethane fraction (2.1 g) was fractionated by Sephadex LH-20 (Ø 0.4 x 38 cm) using methanol as eluent to get 4 fractions (SJEDa - SJEDd). SJEDb (1.181 g) was purified by repeated HPLC using preparative C_{18} reverse-phase column (Atlantis T3, 10 µm, OBD 19 x 250 mm). The gradient elution using water (A) and acetonitrile (B), consisted of a linear gradient for 30 min with 40 to 70% B and 40 min with 70 to 100% B, and isocratic elution for 50 min with 100% B at flow rate 10 ml/min to obtain Compound 4 and SJEDb7 (fatty acids). Water fraction was purified by repeated HPLC using preparative C_{18} reverse-phase column (Atlantis T3, 10 µm, OBD 19 x 250 mm). The gradient elution using water (A) and acetonitrile (B), consisted of a linear gradient for 15 min with 0 to 7% B and 20 min with 7 to 50% B at flow rate 10 mL/min to obtain SJEW1d (salts).

Structure elucidation of compound

The chemical structures of the isolated compounds were determined by NMR and mass spectrometry. For NMR experiment, several analytical NMR techniques including ^{1}H, ^{13}C, COSY, HSQC and HMBC were used. The NMR spectra were measured in DMSO (d_6), methanol (CD_3OD), or deuterium oxide (D_2O) with Varian NMR system (Varian, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. The chemical shifts are reported in parts per million (ppm) from tetramethylsilane. Residual solvent signals (CD_3OD at 3.3 ppm, ^{1}H, and 49.0 ppm, ^{13}C; DMSO-d_6 at 2.49 ppm and 39.5

ppm, respectively) were considered as internal reference signal for calibration. The observed chemical shift values (δ) were given in ppm and the coupling constant (J) in Hz.

For GC/MS determinations, a system combining a GC 7890A with a quadrupol MS 5975C (Agilent Technologies, Waldbronn, Germany) was used. The injection volume was 2 µL (splitless injection; injection port temperature 250°C). A DB-Wax column (30 m x 0.251 mm i.d., 0.25 µm film thickness) from Agilent was used. Carrier gas was helium. The temperature of the column was started at 100°C for 1 min, raised to 180°C at 20°C/min, raised to 250°C at 2°C/min and held for 10 min. The carrier gas was helium at liniear flow rate of 1 mL/min. All spectra were scanned within the range *m/z* 50-700.

ABTS radical cation scavenging activity

$ABTS^{\cdot+}$ scavenging activity of extracts or fractions was determined according to the method described by Re et al. (1999) and Li et al. (2007) with slight modifications. $ABTS^{\cdot+}$ was produced by reacting 7 mM aqueous ABTS solution with 2.45 mM potassium persulfate in the dark at room temperature for 16 h and used within two days. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.050 at 734 nm. Crude extracts and fractions were appropriately diluted with DMSO. A volume of 1.9 mL of diluted $ABTS^{\cdot+}$ solution was added to 50 µL of each sample dilution, and the absorbance at 734 nm was recorded exactly after 6 min at room temperature. The percentage of scavenged $ABTS^{\cdot+}$ was calculated using the following equation: Scavenged $ABTS^{\cdot+}$ (%) = $100(1–(A_S–A_B)/A_C)$, where A_S is the absorbance of sample (50 µL of sample dilution plus 1.9 mL of diluted $ABTS^{\cdot+}$ solution), A_B is the absorbance of blank (50 µL of sample dilution plus 1.9 mL of ethanol), and A_C is the absorbance of control (50 µL of DMSO plus 1.9 mL of the diluted $ABTS^{\cdot+}$ solution). The whole experiment was conducted in triplicate.

Tyrosinase inhibition assay

The tyrosinase activity, using L-DOPA as substrate, was measured according to the method of Kubo and Kinst-Hori (1998) with slight modifications. Briefly, 2.8 mL of a 0.54 mM L-DOPA in 0.1 M phosphate buffer (pH 6.8) was incubated at 25°C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of the mushroom tyrosinase (1380 units/mL) were added to the mixture. The reaction mixture was incubated at 25^{o}C for 0.5 min. The absorbance was measured at 475 nm using Jasco V-530 UV/VIS Spectrophotometer (JASCO Corporation, Tokyo, Japan). Tyrosinase inhibitory activity was calculated using the formula: Inhibitory activity (%) = $[(A_C–(A_S–A_B))/A_C] \times 100$, where A_C is the absorbance of control, A_S is the absorbance of mixture, and A_B is the absorbance of blank. The standard tyrosinase inhibitors, kojic acid and ascorbic acid, were employed as positive control.

Results and discussion

Structure elucidation

The crude EtOH extract of *S. japonicus* was subjected to activity-guided fractionation using solvent partition and column chromatography on Sephadex LH-20 and preparative HPLC. The extraction and isolation scheme is shown in Fig. 1. Structures of these metabolites were deduced using NMR (^{1}H, ^{13}C, COSY, HSQC, and HMBC) and MS analysis and by comparison of their spectroscopic data with those reported in the literature (when available).

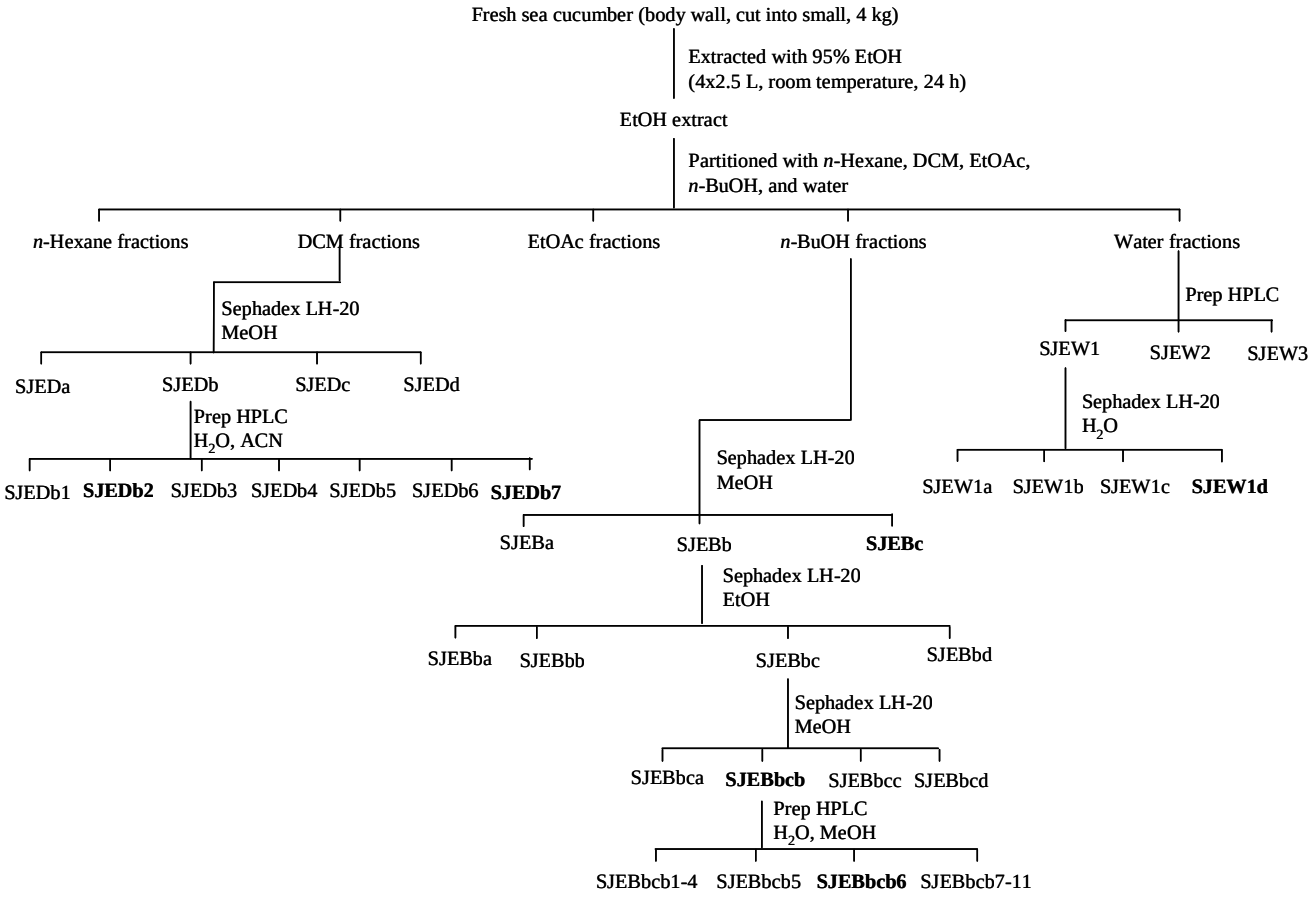

Fig. 1. Isolation scheme for antioxidants and tyrosinase inhibitors from sea cucumber *S. japonicus* body wall.

Compound 1: colorless solid, the molecular formula of the compound was determined to be $C_{10}H_{13}N_5O_4$ (MW = 267.241). ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6) of the compound were shown in Table 1. The ^{1}H NMR spectrum of compound 1 showed two aromatic signals at δ 8.13 (1H, s) and 8.35 (1H, s) which belong to the two protons in the purine nucleus. The other spin system stands for the ribose sugar and includes the anomeric proton at δ 5.87. The ^{1}H-^{1}H COSY spectra of compound 1 enabled the complete assignment of all proton signals as indicted in Table 1 and were consistent with the structure assigned. Finally the ^{13}C-NMR spectral data of compound 1 confirmed the structure of compound 1. Compound 1 was identified as adenosine. The structure was confirmed in comparison with literature data (Ciuffreda et al., 2007).

Table 1. 1D and 2D NMR data for the fraction of compound 1 in DMSO at 500 MHz

Position	δH (m, *J* in Hz)	δC	COSY	HMBC
2	8.35 (s)	139.91		C-5, C-4, C-6
8	8.13 (s)	152.39	H-1’	C-4, C-6, C-5
NH_2	7.34 (s)			C-5, C-4, C-2
1’	5.87 (d, 6.2)	87.86	H-2’, H-8	C-3’, C-2’, C-4’, C-8, C-4
2’-OH	5.48 (d, 6.2)		H-2’	C-3’, C-2’, C-1’
5’-OH	5.45 (dd, 4.5, 7.2)		H-5’a, H-5’b	C-4’, C-5’
3’-OH	5.23 (d, 4.5)		H-3’	C-3’, C-2’, C-1’
2’	4.6 (ddd, 6.2, 6.2, 5.1)	73.47	H-1’, OH-2’, H-3’	C-4’, C-1’
3’	4.15 (m)	70.67	OH-3’, H-2’, H-4’	C-1’
4’	3.96 (q, 3.5, 3.5, 3.2)	85.89	H-3’, H-5’ab	C-3’
5’a	3.66 (dt, 4.3, 4.3, 12.1)	61.66	H-4’, OH-5’, H-5’b	C-3’, C-4’
5’b	3.54 (ddd, 3.6, 7.3, 12.0)	61.66	H-4’. OH-5’, H-5’a	C-3’

Table 2. 1D and 2D NMR data for the fraction of compound 2 in D_2O at 500 MHz

Position	δH (m, *J* in Hz)	δC[a]	COSY	HMBC
1	4.92 (d, 3.81)	97.70	H-2	C-7,C-5,C-3
2	3.54 (dd, 3.8, 9.81)	71.08	H-1,H-3	C-1,C-3
3	3.68 (d, 9.8)	73.02	H-2, H-4	C-4,C-2
4	3.38 (dd, 9, 9)	69.50	H-3, H-5	C-5,C-3
5	3.67 (d, 1.54)	71.49	H-4	C-4,C-6
6a	3.84 (d, 2.12)	60.40		C-4,C-5
6b	3.73 (s)	60.40		
7a	3.78 (d, 2.53)	63.71		
7b	3.55 (m)	63.71		C-1

[a]Signals were assigned by HMBC and HSQC experiments

Table 3. 1D and 2D NMR data for the fraction of compound 3 in D_2O at 500 MHz.

Position	δH (m, *J* in Hz)	δC[a]	COSY	HMBC
1	-	126.7		
2	7.18 (d, 8.66)	130.6	H-3,H-7a	C-4,C-5,C-6,C-7
3	6.88 (d, 8.66)	115.7	H-2	C-1,C-3,C-4
4	-	154.4		
5	-	115.7		
6	-	130.6		
7a	3.19 (dd, 5.17, 9.62)	35.4	H-8,H-7b	C-1,C-2,C-8,C-9
7b	3.04 (dd, 6.78, 7.83)		H-8,H-7a	C-1,C-2,C-8,C-9
8	3.92 (dd, 2.70, 5.13)	55.9	H-7a,H-7b	C-1,C-7,C-9
9	-	174.5		

[a]Signals were assigned by HMBC and HSQC experiments

Compound 2: colorless solid, the molecular formula of the compound was identified to be $C_8H_{16}O_6$ (MW = 208.209). ^{1}H NMR (D_2O, 500 MHz) and ^{13}C NMR (D_2O) were shown in Table 2. Compound 2 was elucidated as ethyl-α-D-glucopyranoside. The structure was confirmed in comparison with literature data (Teague et al., 2004).

Table 4. 1D and 2D NMR data for the fraction of compound 4 in CD_3OD at 500 MHz

Position	δH (m, *J* in Hz)	δC[a]	COSY	HMBC
1	7.20 (bt, 0.9)	122.9	H-9	C-2,C-3,C-8
2	-	110.9		
3	-	126.5		
4	7.55 (dd, 0.49, 8.46)	121.2	H-5	C-6
5	7.15 (dd, 1.75, 8.46)	123.3	H-4, H-7	C-3,C-6
6	-	114.9		
7	7.50 (dd, 0.48, 1.76)	114.6	H-5	C-6,C-5,C-3
8	-	138.7		
9	3.42 (d, 0.9)	24.2	H-1	C-11,C-10,C-2,C-3
10	-	74.4		
11	-	173.2		

[a]Signals were assigned by HMBC and HSQC experiments

Compound 3: colorless solid, the molecular formula of the compound was identified to be $C_8H_{16}O_6$ (MW = 181.189). ^{1}H NMR (D_2O, 500 MHz) and ^{13}C NMR (D_2O) were shown in Table 3. Compound 3 as elucidated as L-tyrosine.

Compound 4: pale yellow, the molecular formula of the compound was determined to be $C_{11}H_{10}BrNO_3$ (MW = 284.106). ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD) were shown in Table 4. The ^{1}H-NMR spectrum showed the typical coupling pattern of phenyl ring system with a *meta* coupling proton H-7 at δ 7.50 (1H, dd), an *ortho* coupling proton H-4 at δ 7.55 (1H, dd), and an *ortho-meta* coupling proton H-5 at δ 7.15 (1H, dd). Furthermore, it showed a downfield at δ 7.20 (1H, bt), characteristic of the indole ring proton. Compound 4 was proposed as 3-(6-bromo-1*H*-indol-3-yl)-2-hydroxy-propionic acid.

Structures of compound (**1**) adenosine, (**2**) ethyl-α-D-glucopyranoside, (**3**) L-tyrosine, and (**4**) 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid were shown in Fig 2.

SJEDb7: pale yellow oil, from NMR and GC-MS data this fraction was identified as

fatty acids.

SJEW1d: white, from NMR and atomic absorption spectrometry analysis was identified as salts with composition: Na (81.3%), Mg (16.3), K (1.6%), and Ca (0.8%).

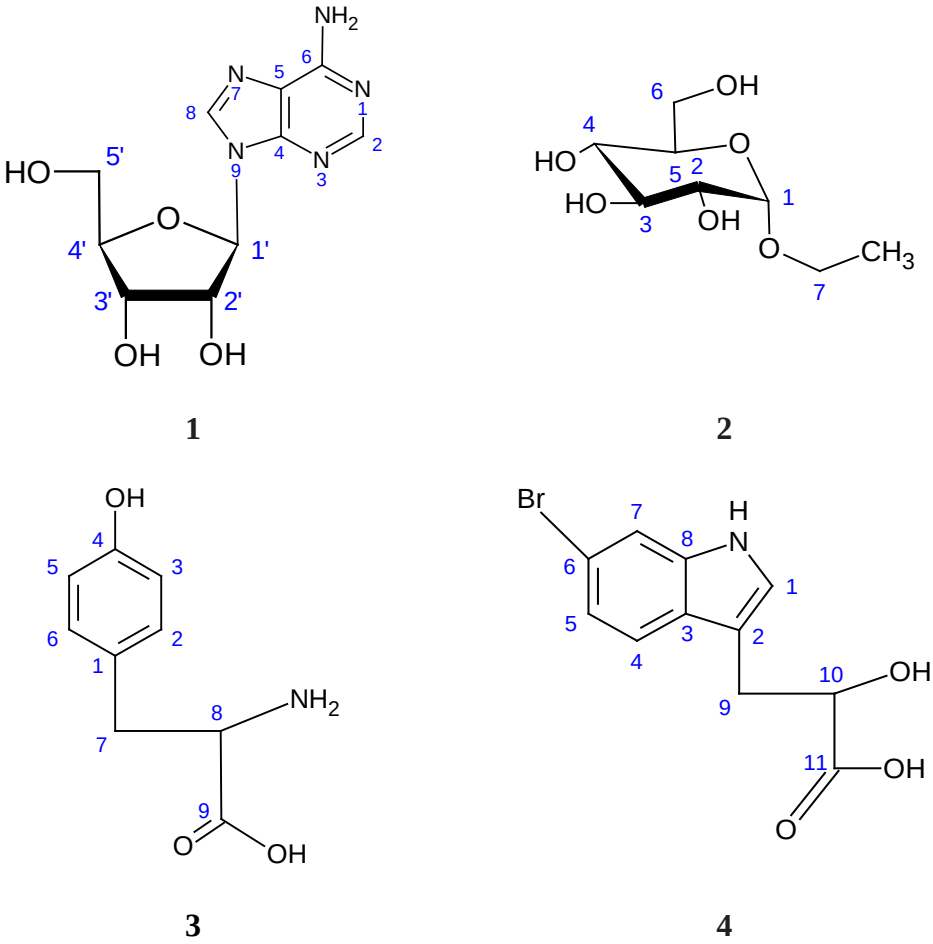

Fig. 2. Structures of adenosine (**1**), ethyl-α-D-glucopyranoside (**2**), L-tyrosine (**3**), and 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (**4**).

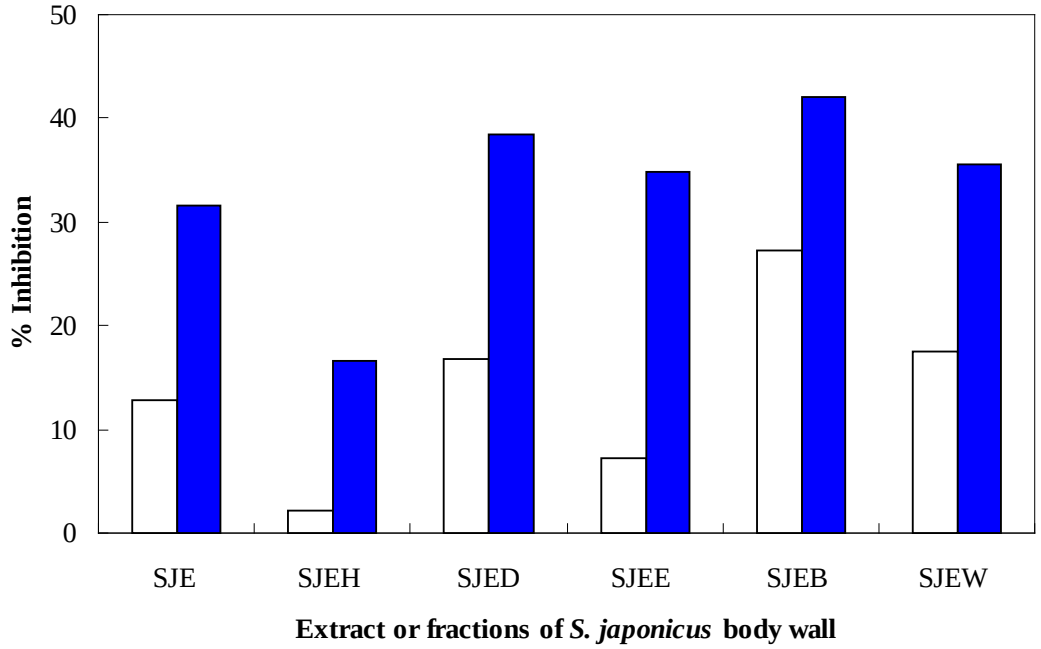

Fig. 3. Antioxidant and tyrosinase inhibitory activities of ethanol extract from *S. japonicus* body wall and its fractions. (□ Scavenging of $ABTS^{\cdot+}$, ■ Inhibition of mushroom tyrosinase). SJE = ethanol extract, SJEH = hexane fraction, SJED = dichloromethane fraction, SJEE = ethyl acetate fraction, SJEB = butanol fraction, SJEW = water fraction).

Antioxidant activity

The ethanol extract was partitioned into fifth parts and their antioxidant activities are summarized in Fig. 3. The activity of fractions or purified compounds was measured by $ABTS^{\cdot+}$ scavenging assay and the results were compared with that of a well-known reference antioxidant, ascorbic acid (Table 5). IC_{50} values of of SJEBc (compound 1), SJEBbcb, and SJEDb2 were 212, 195, and 106 μg/mL, respectively. SJEBc (compound 1 was identified as adenosine) and SJEBbcb showed a moderate $ABTS^{\cdot+}$ scavenging activity, whereas SJEDb2 showed more potent antioxidant activity. SJEBbcb fraction was isolated to obtain ethyl-α-D-glucopyranoside (compound 2) and L-tyrosine (compound 3). Yokoi et al. (1995) reported that adenosine scavenged reactive oxygen species especially hydroxyl radicals (OH). The active site for scavenging OH might be exist between N-1 and C-6. Ethyl-α-D-

glucopyranoside (compound 2) is first reported in sea cucumber. Ethyl-α-D-glucopyranoside have been isolated from sake (Imanari and Tamura, 1971), however, their antioxidant activity have not been reported. L-tyrosine has been reported to have an effect on ABTS radical cation scavenging activity (Gülçin, 2007). In this study, antioxidant activity of SJEDb2 fraction showed comparable with ascorbic acid. SJEDb2 fraction was identified to get 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (compound 4) as a novel compound. Antioxidant activity of bromoindole derivatives has been reported. The bromoindole, 6-bromo-5-hydroxyindole, isolated from *Drupella fragum* was found to have a strong antioxidative potency as butylated hydroxytoluene (BHT) (Ochi et al., 1998).

Tyrosinase inhibitory activity

From 4 kg of fresh *S. japonicus*, 1.70 g of orange color extract was obtained. The extract was partitioned into fifth parts and their bioassay results are summarized in Fig. 2. In butanol part, 42.11% of tyrosinase inhibitory activity was found. Tyrosinase inhibitory activity of compounds or fractions using L-DOPA as substrate was examined. Each compound or fraction was assayed at different concentrations and their relative activities were expressed as IC_{50} values. As shown in Table 5, the IC_{50} values of SJEBc (compound 1), SJEBbcb, SJEDb7, and SJEW1d were 191, 126, 250, and 127 μg/mL, respectively. Their activities are lower than those of kojic acid and ascorbic acid, as well-known tyrosinase inhibitors. SJEBc was identified as adenosine (compound 1). Based on literature, tyrosinase inhibitory activity of adenosine isolated from pollen of *Crocus sativus* L. (Li and Wu, 2002) was in agreement with our results. Kaemferol glycoside has been isolated from *Lamium amplexicaule* L. and its tyrosinase inhibitory activity (IC_{50} = 194 μg/mL) was comparable with adenosine in this study (Nugroho et al., 2009). SJEBbcb fraction was purified to obtain ethyl-α-D-glucopyranoside (compound 2) and L-tyrosine (compound 3). Ethyl-α-D-

glucopyranoside (compound 2) is first reported in sea cucumber. Ethyl-α-D-glucopyranoside have been isolated from sake (Imanari and Tamura, 1971), however, their tyrosinase inhibitory activity have not been reported. SJEDb7 was identified as fatty acids. Ando et al. (2004) reported that fatty acids fatty acids can act as intrinsic factors that modulate the proteasomal degradation of tyrosinase. SJEW1d fraction was identified as salts and the major component of this salts is Na. Tyrosinase inhibitory activity of NaCl has been reported and the tyrosinase inhibitory activity of salts on this study (IC_{50} = 127 μg/mL) was higher than NaCl (IC_{50} = 1461 μg/mL) (Park et al., 2005).

Table 5. Antioxidant and tyrosinase inhibitory activities of fractions or compounds isolated from *S. japonicus* body wall.

Fractions/ compounds	$ABTS^{\cdot+}$ radical scavenging activity (IC_{50}, μg/mL (mM))	Tyrosinase inhibitory activity (IC_{50}, μg/mL (mM))
SJEBc (1)	212 (0.793)	191 (0.714)
SJEBbcb (2,3)	195	126
SJEDb2 (4)	106 (0.373)	NA[a]
SJEDb7	NA[a]	250
SJEW1d	NA[a]	127
Kojic acid	ND[b]	4.25 (0.030)
Ascorbic acid	9.3 (0.053)	21 (0.119)

[a]Not active
[b]Not determined

Conclusions

Four compounds, fatty acids, and salts were isolated from *Stichopus japonicus* and identified as adenosine (1), ethyl-α-D-glucopyranoside (2), L-tyrosine (3), 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4), fatty acids, and salts. Adenosine (1), ethyl-α-D-glucopyranoside (2), L-tyrosine (3), and 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic

acid (4) are responsible for antioxidants. Adenosine (1) and ethyl-α-D-glucopyranoside (2) are also responsible for tyrosinase inhibitors. On the other hand, fatty acids and salts are only responsible for tyrosinase inhibitors. Ethyl-α-D-glucopyranoside (2) and 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4) are first reported in sea cucumber. Furthermore, 3-(6-bromo-1*H*-indole-3-yl)-2-hydroxy-propionic acid (4) was identified as a novel compound.

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