Antioxidant and antidiabetic activities of vanadium binding proteins purified from the sea squirt *Halocynthia roretzi*

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Abstract Sea squirts accumulate vanadium compounds with potent antidiabetic activity, which are involved in immune defense. In this study, vanadium concentrations of fresh blood plasma, intestine, and muscle of the sea squirt *Halocynthia roretzi* were 6.3, 3.7 and 2.1 mg/kg respectively. Two vanadium binding proteins (VBPs) from blood plasma and intestine were purified through $(\text{NH}_4)_2\text{SO}_4$ precipitation, and DEAE-Sepharose ion exchange and Sephacryl S-200 HR gel filtration chromatography, in that order. The purity and yield of the intestine and blood plasma vanadium binding proteins, VBP_{intestine} and VBP_{blood plasma}, were 13.4 folds and 7.1%, and 20.9 folds and 6.8%, respectively. There were two protein bands on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weights of 24.3 and 68.8 kDa and one with 96.7 kDa on the native-PAGE of VBP_{blood plasma}, whereas only one protein band of VBP_{intestine} on the SDS-PAGE with 26.5 kDa. Antioxidant activities of VBPs were lower than that of ascorbic acid. Both VBPs exerted strong inhibitory activity against *Saccharomyces cerevisiae* and mild against *Bacillus stearothermophilus* and rat intestinal α -glucosidase. IC_{50} values of VBP_{intestine} and VBP_{blood plasma} against *S.*

cerevisiae α -glucosidase were 28.34 and 12.60 $\mu\text{g/ml}$, respectively. The K_m , V_{max} , k_{cat} , and k_{cat}/K_m values of VBP_{intestine} and VBP_{blood plasma} were 4.29, 0.036, 6.58 and 1.53×10^3 , and 7.63 mM, 0.057 mM/min, 10.41 s^{-1} and $1.36 \times 10^3 (\text{M sec})^{-1}$, respectively. There was a synergistic interaction between VBP_{blood plasma} and VBP_{intestine} on rat intestinal α -glucosidase inhibitory activity.

Keywords Sea squirt · Vanadium binding proteins · Intestine · Blood plasma · Antidiabetic · Antioxidant

Introduction

Vanadium is a transition metal widely distributed in the biosphere and exists in a variety of compounds with oxidation states ranging from -3 to $+5$. Among them, V^{III} , V^{IV} , and V^{V} are of biological relevance and known to be presented in the active sites of several vanadium-dependent enzyme systems (Pessoa et al. 2015). Moreover, vanadium (V) compounds known as vanadates have significant enzyme inhibitory activities due to their closely related physicochemical properties with phosphate in biological systems (Crans et al. 2004). Vanadium compounds attracted much attention because of their high potential as antidiabetic agents due to insulin mimetic activity (Peters et al. 2003).

The accumulation of vanadium with unusual amounts in ascidians attracted many scientists toward comprehensive research related to the role of vanadium in marine organisms (Yoshihara et al. 2008). The concentration of vanadium in several ascidian species was determined, in which *Ascidia gemmata* recorded the highest concentration of 350 mM which was 10^7 times higher than that of sea water (Ueki and Michibata 2011). The vanadium concentrations

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in the internal tissues and blood of the solitary ascidian *Phallusia nigra* were higher than that of the whole tunic, and at low pH values, vanadium metabolites exerted chemical defense in the ascidian (Odate and Pawlik 2007). Several proteins are known to be involved in the accumulation of vanadium in ascidians (Yoshinaga et al. 2007). Among them, vanabin family in blood cells (vanadocytes) and coelomic fluid of the ascidian *Ascidia sydneiensis samea* gained much attention due to their selective binding ability for vanadium (Ueki et al. 2003, 2008). A vanadium binding protein of the sea cucumber *Apostichopus japonicus* intestine had adipocyte differentiation inhibitory effect which was mediated by activating the WNT/ β -catenin pathway (Liu et al. 2015a). Inorganic vanadium (IV) and (V) complexes and synthetic vanadyl complexes with organic ligands are known to have antioxidant activities (Francik et al. 2011; Wazalwar and Bhawe 2012). But the antioxidant activities of vanadium binding proteins have not been investigated so far.

The sea squirt *Halocynthia roretzi* is a popular sea food in Korea and Japan, in which many bioactive compounds have been extracted. Among them, carotenoids such as halocynthiaxanthin and fucoxanthinol of *H. roretzi* are reported to exhibit antiproliferative activity against human leukemia, breast and colon cancer cells (Konishi et al. 2006). However, there is no comprehensive study on vanadium binding proteins of the sea squirt *H. roretzi* so far. Therefore, the objectives of this study were to determine the vanadium concentrations in the tissues of *H. roretzi*, purify vanadium binding proteins and analyze their antidiabetic and antioxidant activities.

Materials and methods

Materials

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ABTS [2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid] diammonium salt, potassium ferricyanide, potassium, ferric chloride, *p*-nitrophenyl- α -D-glucopyranoside (*p*NPG), phenylmethane sulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), trisaminomethane hydrochloride (Tris-HCl) *S. cerevisiae* and *B. stearotheophilus* α -glucosidase and rat intestinal acetone powder were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE Sepharose fast flow and Sephacryl S-200 HR gels were purchased from GE Healthcare (Uppsala, Sweden). All other chemicals used in this study were of analytical grade.

Sample preparation

Fresh sea squirt *H. roretzi* obtained from a local fishery market (Gangneung, Korea) packed on ice was transported to the laboratory within 1 h. Blood plasma, intestine and muscle tissue of the sea squirt were separated, immediately deep frozen (at -80°C) and stored at -25°C until use.

Determination of vanadium concentration

Samples (0.2 g) in a Kjeldahl flask were mixed with 10 ml of water and 2 ml of nitric acid. This mixture was gently heated for 3–5 min. After cooling, 5 ml of sulfuric acid was added and heated again until the yellow color became colorless. After cooling down to room temperature, 30 ml of water and 10 ml of saturated ammonium hydroxide solution were added, and then heated until the white smoke occurred. Ten ml of the decomposed solution was mixed with 2 ml of 25% ammonium citrate and 2 ml of bromothymol blue, neutralized with aqueous ammonia, and then mixed with 2 ml of 40% ammonium sulfate. Afterwards, 1 ml of 10% diethyl dithiocarbamic acid was added and mixed well. After adding 20 ml of chloroform, it was vigorously shaken and then the chloroform layer was separated. This was repeated with 20 ml of chloroform and then the two chloroform layers were combined, and heated at 80°C using a hot plate. Afterwards, 7 ml of nitric acid and 1 ml of hydrogen peroxide was added to the residue and heated again on the hot plate until completely dried. The residue was redissolved in 10 ml of 1 N nitric acid, which was used as the test solution. The test solution was injected into the ICP-OES spectrophotometer (ICP-OES optima 8300; Perkin Elmer, Waltham, MA, USA). Vanadium concentrations were calculated based on the standard vanadium concentration.

The lyophilized powder of fresh blood plasma, intestine and muscle were dissolved in ultrapure water, dialyzed to remove inorganic vanadium using a dialysis tube with a molecular weight cut off of 3.5 kDa at 4°C for 144 h, in which the ultrapure water was changed every 12 h, and then lyophilized again. The organic vanadium concentrations of the samples were determined using ICP-OES (Perkin Elmer) according to the above mentioned method.

Extraction of vanadium binding proteins

The extraction of VBPs from blood plasma was carried out according to the method of Yoshihara et al. (2005) with slight modification. Blood plasma was separated from coelomic fluid after removing the blood cells by centrifuging at $1000\times g$ at 4°C for 10 min. Then 500 mM Tris-HCl (pH 8.0) was added (one-ninth of sample volume) and mixed well. The proteins in the blood plasma

were precipitated with the 80% $(\text{NH}_4)_2\text{SO}_4$ at 4 °C for 20 h. The blood plasma proteins were obtained by centrifuging at $17,400\times g$ and 4 °C for 30 min and were resuspended in 200 ml of 20 mM NaH_2PO_4 (pH 7.2) containing 500 mM NaCl. Afterwards, it was dialyzed against a 50 mM Tris–HCl buffer containing each 1 mM of PMSF and EDTA at pH 7.4 and 4 °C for 24 h. The extraction of VBPs from intestine and muscle was carried out according to the method of Liu et al. (2015a, b) with slight modification. Each intestine and muscle of sea squirt was collected and homogenized in five volumes (ml per gram wet weight) of a homogenizing buffer (200 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0). The homogenized solution was precipitated with the 30% $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at $17,400\times g$ and 4 °C for 10 min. Afterwards, the supernatant obtained by centrifugation was precipitated with the 80% $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at $17,400\times g$ and 4 °C for 30 min. The 80% $(\text{NH}_4)_2\text{SO}_4$ precipitate was redissolved in five volumes (ml per gram wet weight) in Tris–HCl buffer (50 mM, pH 8.0) and dialyzed overnight at 4 °C against the same buffer containing each 1 mM PMSF and EDTA, and then lyophilized.

Purification of vanadium binding proteins

The ammonium sulphate precipitates of blood plasma (1.8 g) and intestine (1.4 g) were dissolved in 5 ml of 20 mM NaH_2PO_4 (pH 7.2). Then it was filtered through a 0.45 μm membrane and loaded to a DEAE Sepharose fast flow ion exchange column (1.6×30.0 cm) pre-equilibrated with 50 mM Tris–HCl (pH 7.4). Vanadium binding proteins were eluted with a linear gradient of 0–400 mM NaCl in the same buffer at a flow rate of 1.0 ml/min, in which first 30 min was eluted with only equilibrating buffer and thereafter changing the elution buffer to 100, 200 and 400 mM NaCl concentration gradient at every 90 min. Fractions were collected by a fraction collector (6 ml/tube) (FC204; Gilson Inc., Middleton, WI, USA). The protein fractions with vanadium were further purified using Sephacryl S-200 HR column (2.6×70.0 cm) with 50 mM Tris–HCl buffer (pH 8.0) at a flow rate of 0.7 ml/min. Protein fractions (6 ml/tube) containing vanadium (VBP_{blood plasma}: fraction 18–36; VBP_{intestine}: fraction 25–42) were pooled, lyophilized and stored at – 25 °C until use.

Protein concentration

Protein concentrations in the eluted fractions by column chromatography were determined using the Bradford method (Kruger 1994). Bovine serum albumin (BSA) was

used as the standard and the absorbance at 750 nm was measured.

Electrophoresis

The purified vanadium binding proteins were applied to SDS-PAGE and native-PAGE on 10 and 8% polyacrylamide gel, respectively according to the method of Laemmli (1970) and Arndt et al. (2012). The electrophoresed gel was stained using 0.1% Coomassie Blue R-250 and was destained in a destaining solution (40% methanol and 10% acetic acid).

Antioxidant activity

DPPH radical scavenging activity

The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined according to the method of Kaur et al. (2012). Five hundred μl of sample was mixed with 0.5 ml of 100 mM DPPH in methanol and incubated at room temperature for 30 min in the dark. The absorbance at 517 nm was read against a blank. The inhibition of DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = $[(\text{Ac} - \text{As})/\text{Ac}] \times 100$, where Ac was the absorbance of the control and As was the absorbance of the sample. Ascorbic acid was used as the positive control. The inhibitor concentration in the reaction mixture required to inhibit 50% of the enzyme is defined as the IC_{50} value.

ABTS radical scavenging activity

The 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical scavenging activity was determined according to the method of Akkari et al. (2016) with slight modification. ABTS (7 mM) was mixed with 2.45 mM potassium persulfate (1:1, v/v), and stood for 12–16 h at room temperature in the dark before use. The ABTS solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. Five hundred μl of sample was mixed with 1.5 ml of ABTS solution and then incubated at room temperature for 20 min. The absorbance at 734 nm was read against a blank. The percentage of scavenged ABTS was calculated using the following equation:

ABTS scavenging activity (%) = $[(\text{Ac} - \text{As})/\text{Ac}] \times 100$.

Ascorbic acid was used as the positive control.

FRAP assay

Ferric reducing antioxidant power assay was performed according to the method of Oyaizu (1986) with slight modification. Two hundred μl of sample was mixed with 250 μl of sodium phosphate buffer (0.2 M, pH 6.6) and 500 μl of 1% potassium ferricyanide, and then incubated at 50 °C for 20 min. After the addition of 500 μl of 10% TCA, the mixture was centrifuged at $1000\times g$ for 10 min. Afterwards, 500 μl of supernatant was mixed with 100 μl of 0.1% ferric chloride (freshly prepared), diluted with 500 μl of distilled water, and reacted for 10 min. The absorbance at 700 nm was read against a blank. Ascorbic acid was used as the positive control. An increase in the absorbance of the reaction mixture indicates an increase in the reducing power. The effective concentration (EC_{50}) is defined as the concentration of the inhibitor that gives the half-maximal response. EC_{50} of each test sample at absorbance of 0.5 was calculated.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Kawee-ai et al. (2013). One hundred μl of the sample solution was added to 100 μl of 0.1 M phosphate buffer (pH 5.0) and mixed in a 96-microwell plate. Then, 20 μl of 2 mM hydrogen peroxide was added and incubated at 37 °C for 5 min. Thirty μl of 1.25 mM ABTS and 1.0 unit/mL peroxidase were added to the reaction mixture and followed by incubation at 37 °C for 10 min. The absorbance at 405 nm was read with a microplate reader (BioTek Instruments, Winooski, VT, USA). The ability to scavenge the hydrogen peroxide radical was calculated by the following equation;

$$\% \text{ inhibition} = [\text{A}_\text{C} - \text{A}_\text{S}] / \text{A}_\text{C} \times 100.$$

Antidiabetic activity

α -Glucosidase inhibitory activity

Saccharomyces cerevisiae and *B. stearothermophilus* α -glucosidase inhibitory activities were determined according to the method of Nguyen and Kim (2015). A reaction mixture containing 2.2 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of 0.25 U/ml α -glucosidase in 0.01 M phosphate buffer (pH 7.0), and 0.1 ml of sample was incubated at 37 °C for 5 min. One hundred μl of 3 mM *p*NPG in the same buffer was added to the above mixture and incubated at 37 °C for 30 min. Afterwards, the reaction was stopped by adding 1.5 ml of 0.1 M Na_2CO_3 . α -Glucosidase activity was quantified by measuring the absorbance at 405 nm. Acarbose was used as the positive control. The inhibition

percentage against α -glucosidase by the sample was calculated by the following equation;

$$\% \text{ inhibition} = [\text{A}_\text{C} - \text{A}_\text{S}] / \text{A}_\text{C} \times 100.$$

The inhibitor concentration in the reaction mixture required to inhibit 50% of the enzyme is defined as the IC_{50} value.

Rat intestinal maltase and sucrase activity

Rat intestinal maltase assay was carried out according to the method of Nguyen and Kim (2015). Rat intestinal acetone powder (1.0 g) was suspended in 3 ml of 0.9% saline. This suspension was sonicated three times at 4 °C for 30 s, and then centrifuged at $1200\times g$ for 30 min. The resulting supernatant after centrifugation was used as maltase or sucrase solution. Fifty μl of the sample solution was incubated with 100 μl of rat intestinal maltase solution at 37 °C for 10 min. And then, 50 μl of 5 mM *p*-nitrophenyl- α -D-glucopyranoside in 0.01 M phosphate buffer (pH 7.0) was added and incubated at 37 °C for 15 min. Absorbance at 405 nm was measured with a microplate reader (BioTek Instruments). Sucrase inhibitory activity was assayed according to the method of Li et al. (2014) with slight modification. Fifty μl of rat intestinal sucrase solution was incubated with 50 μl of sample solution at 37 °C for 10 min. Afterwards, 0.4 mg/ml sucrose in 100 μl of 0.1 M phosphate buffer (pH 7.0) was added to the reaction mixture and incubated at 37 °C for 30 min. The reaction was stopped by heating in a boiling water bath for 10 min. The amount of liberated glucose was measured using glucose oxidase method with Glucose (GO) Assay Kit (Sigma-Aldrich). Acarbose was used as the positive control. The inhibition percentage was calculated as follows;

$$\% \text{ inhibition} = [\text{A}_\text{C} - \text{A}_\text{S}] / \text{A}_\text{C} \times 100.$$

α -Amylase inhibitory activity

α -Amylase inhibitory activity was determined according to the method of Kazeem et al. (2013). Two hundred fifty μl of sample was added to 250 μl of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.5 mg/ml of α -amylase solution. This mixture was incubated at 25 °C for 10 min, and then 250 μl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added and further incubated at 25 °C for 10 min. The reaction was terminated by adding 500 μl of dinitrosalicylic acid (DNS) reagent. The reaction mixture was then incubated in a boiling water bath for 5 min, cooled to room temperature and diluted with 5 ml of distilled water. The absorbance at 540 nm was

measured. Acarbose was used as the positive control. The inhibition percentage was calculated as below;

$$\% \text{ inhibition} = [A_C - A_S] / A_C \times 100.$$

Kinetics of α -glucosidase inhibitors

Saccharomyces cerevisiae α -glucosidase inhibition assay was carried out according to the above mentioned method at inhibitor concentrations of 2 and 8 $\mu\text{g/ml}$. The type of inhibition and kinetic parameters were determined using Lineweaver–Burk plots (Lineweaver and Burk 1934) and secondary replots (Silverman 2002). The kinetic parameters K_m and V_{max} were calculated from the Lineweaver–Burk plot. K_{cat} and K_{cat}/K_m values were calculated based on the K_m and V_{max} values.

Combined effect of the two inhibitors against rat intestinal maltase

The combined effect of the two inhibitors was evaluated using the combination index (CI) according to the following equation, where d1 and d2 are the concentrations of VBP_{blood plasma} and VBP_{intestine} when used in combination. D1 and D2 are the concentrations of each VBP that individually produce the same effect as the combination (d1 + d2) (Martinez-Irujo et al. 1998).

Twenty-five μl of 1 mg/ml VBP_{blood plasma} was mixed with 25 μl of VBP_{intestine} at different concentrations of 200–500 $\mu\text{g/ml}$. The inhibitory effect of the VBP mixtures against rat intestinal maltase was performed according to the above rat intestinal maltase assay method.

Statistical analysis

SPSS for windows (version 23.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Each value was expressed as the mean $\pm$ standard deviation. Differences between the individual groups were assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range *t* test. A value of $p < 0.05$ was considered to represent a statistically significant.

Results and discussion

Vanadium concentrations of the sea squirt

The total vanadium concentrations of the fresh blood plasma, intestine and muscle were 6.3, 3.7 and 2.1 mg/kg, respectively, whereas vanadium concentrations were 5.3, 2.0 and 0.6 mg/kg, respectively (supplementary material). Total and organic vanadium concentrations of blood

plasma were the highest, followed by intestine and muscle, in that order, which was similar to the findings of Liu et al. (2015b). Vanadium concentrations of several sea squirts in the suborders Phlebobranchia and Stolidobranchia of class Ascidiacea were determined by Michibata et al. (1986), in which the total vanadium concentration of the sea squirt *H. roretzi* was 0.6 ng/mg, which was 1/10 times of the total vanadium concentration, 6.3 mg/kg, in this study. This might be due to that accumulation of vanadium in marine organisms is affected by locational and seasonal variations (Liu et al. 2015b) as well as by different species.

Purification of vanadium binding proteins

Ammonium sulfate precipitate of muscle didn't exert any antidiabetic or antioxidant activity at all (data not shown). Therefore, only ammonium sulfate precipitates of the intestine and blood plasma were further purified using DEAE ion exchange and Sephacryl S-200 HR gel filtration chromatography. The purity and yield of the VBP_{intestine} and VBP_{blood plasma} were 13.4 folds and 7.1%, and 20.9 folds and 6.8%, respectively (Table 1). Purification ratio of ammonium sulphate precipitates of both VBP_{intestine} and VBP_{blood plasma} was reduced to 0.9, in which certain amount of vanadium was lost. This might be due to the fact that vanadium is bound to other macromolecules such as lipids (Liu et al. 2016). There were two protein peaks (Peak I and II) on the patterns of ion exchange chromatograms of the intestine and blood plasma (Fig. 1a), where only Peak II of intestine and Peak I of blood plasma contained vanadium of 10.0 and 54.0 mg/kg, respectively. There were two protein peaks, Peak IA and IB, on the patterns of gel filtration chromatograms of the blood plasma Peak I, whereas only one protein peak, Peak IIA of the intestine Peak II (Fig. 1b). However, only Peak IB and IIA contained vanadium of 64.0 and 15.0 mg/kg, respectively. The Peak IIA contained one protein, while Peak IB had 2 proteins on SDS-PAGE with apparent molecular weight of 26.5, and 68.8 and 24.3 kDa, respectively (supplementary material). When Peak IB was further analyzed using native-PAGE, it contained only one protein with an apparent molecular weight of 96.7 kDa. Therefore, it was concluded that Peak IB of VBP_{blood plasma} was a protein dimer, whereas Peak IIA of VBP_{intestine} was a protein monomer.

Antioxidant activity

In this study, the antioxidant activities of the sea squirt VBPs were investigated using several free radicals assays in vitro. Ammonium sulphate precipitates of VBPs were defined as the crude. The scavenging activity of all the crude and the purified VBPs on DPPH radicals increased in

Table 1 Purification of the vanadium binding proteins from sea squirt *H. roretzi*

Purification step	Tissue	Amount (ml)	Protein concentration (mg/ml)	Total protein (mg)	Dried weight (mg)	Organic vanadium concentration (mg/kg)	Total amount of vanadium (µg)	Specific vanadium concentration* (µg/g)	Purification ratio (fold)	Yield (%)
Fresh tissue	Blood plasma	2500	1.03	2575	3600	5.3	19.1	7.4	1	100
	Intestine	2000	0.92	1840	2100	2.0	4.2	2.3	1	100
	Muscle	1000	1.13	1130	3160	0.6	1.9	1.7	1	100
(NH ₄) ₂ SO ₄ precipitate	Blood plasma	200	7.84	1568	1800	6.1	11.0	7.0	0.9	57.6
	Intestine	100	12.08	1208	1400	1.7	2.4	2.0	0.9	57.1
	Muscle	50	14.36	718	980	1.6	1.6	2.2	1.3	84.2
DEAE ion exchange	Blood plasma	90	1.09	98	120	54.0	6.5	66.3	8.9	34.0
	Intestine	66	2.50	165	180	10.0	1.8	10.9	4.7	42.9
Sephacryl S-200 HR	Blood plasma	120	0.07	8.4	20	64.0	1.3	154.8	20.9	6.8
	Intestine	108	0.09	9.7	20	15.0	0.3	30.9	13.4	7.1

*Specific vanadium concentration was calculated based on amount of organic vanadium and total protein

**Yield was calculated based on the total amount of vanadium

Mean values are significantly different at $p < 0.05$

Fig. 1 **a** DEAE ion exchange chromatography pattern and **b** Sephacryl S-200 HR gel filtration chromatography pattern of vanadium binding proteins

a dose-dependent manner. IC_{50} values were calculated for the DPPH, ABTS and hydrogen peroxide radical scavenging assays since there was a reduction in the color complexes formed, whereas effective concentration (EC_{50}) at 0.5 absorbance was calculated for FRAP assay as there was an increased production of color complex with increasing antioxidant activity. IC_{50} values of the VBP_{blood plasma} and VBP_{intestine} on DPPH radical scavenging activity were 367.38 and 119.29 µg/ml, respectively, whereas that of ascorbic acid was 41.76 µg/ml. Wazalwar and Bhawe (2012) reported the DPPH radical scavenging activity of inorganic vanadium (IV) complexes,

in which antioxidant activity of vanadium compounds was strong similar to that of the ascorbic acid, the positive control. Therefore, vanadium binding proteins might have lower antioxidant activity compared with the inorganic vanadium compounds. ABTS radical scavenging activity was similar to that of DPPH assay, in which IC_{50} values of the two VBPs were much higher than that of the positive control. There was no hydrogen peroxide scavenging activities in the crude VBPs, whereas VBP_{intestine} and VBP_{blood plasma} had activity in a dose-dependent manner with IC_{50} values of 230.70 and 301.80 µg/ml, respectively. Reducing power of the crude and purified VBPs increased

with increasing concentrations. The EC_{50} values of $VBP_{\text{blood plasma}}$ and $VBP_{\text{intestine}}$ were 210.78 and 146.82 $\mu\text{g/ml}$, respectively, which was higher than 127.03 $\mu\text{g/ml}$ of ascorbic acid. Based on the IC_{50} and EC_{50} values, $VBP_{\text{intestine}}$ had higher antioxidant activity compared to $VBP_{\text{blood plasma}}$. Furthermore, it was observed that the two purified VBPs had higher antioxidant activity than the crude extract. However, the antioxidant activities of two VBPs were not very strong compared to that of ascorbic acid, the positive control (Table 2).

α -Glucosidase inhibitory activity

The inhibitory activity of sea squirt VBPs against *S. cerevisiae* and *B. stearothermophilus* α -glucosidases was investigated using *p*-nitrophenyl- α -D-glucopyranoside (*p*NPG) as a substrate. IC_{50} values of $VBP_{\text{blood plasma}}$ and $VBP_{\text{intestine}}$ against *S. cerevisiae* α -glucosidase were 12.60 and 28.34 $\mu\text{g/ml}$, respectively, which was much lower than 1625.07 $\mu\text{g/ml}$ of acarbose, the positive control (Table 3). IC_{50} values of $VBP_{\text{blood plasma}}$ and $VBP_{\text{intestine}}$ against *B. stearothermophilus* α -glucosidase were 8.64 and 12.02 $\mu\text{g/ml}$, respectively, which was much higher than 0.07 $\mu\text{g/ml}$ of acarbose. The two vanadium binding proteins strongly inhibited yeast α -glucosidase, but did mildly bacterial α -glucosidase compared to the positive control. None of the VBPs inhibited rat intestinal sucrase, but both did mildly rat intestinal maltase. This is an important finding because rat intestinal maltase is a mammalian α -glucosidase. Hence, the two VBPs might have positive effect on the human α -glucosidase as well. An inorganic vanadium compound in +5 oxidation state, ammonium metavanadate (NH_4VO_3), was also investigated for its α -glucosidase inhibitory activity. However, the inorganic vanadium compound didn't have α -glucosidase inhibitory activity. The toxic effects of heavy metals are known to inhibit certain enzyme activities (Pritsch et al. 2006). Heavy metal ions are strongly bound to sulfhydryl groups of proteins which cause the structural changes and enzymatic activities

of proteins (Hodson 1988). The enzymes lactate dehydrogenase, malate dehydrogenase and cytochrome oxidase were inhibited by the heavy metals accumulated in the Mediterranean mussel, *Donax trunculus* (Mizrahi and Achituv 1989). Vanadates are competitive inhibitors of (Na/K)-ATPase as well as bovine low molecular weight protein tyrosine phosphatases (PTPs), in which vanadates form covalent intermediates with the enzyme (Bellomo et al. 2016). However, ammonium metavanadate didn't have α -glucosidase inhibitory activity in this study.

α -Amylase inhibitory activity

α -Amylase plays a role in the starch digestion. Therefore, the inhibition of α -amylase is a strategy for the management of diseases such as diabetes and obesity. The two VBPs didn't inhibit α -amylase at 0.1 mg/ml, whereas the positive control acarbose had an IC_{50} value of 0.014 mg/ml.

Kinetics of *S. cerevisiae* α -glucosidase inhibition

Enzyme kinetics of *S. cerevisiae* inhibition by the two VBPs were carried out at different concentrations of *p*NPG. Based on the Lineweaver-Burk plots, $VBP_{\text{blood plasma}}$ was a competitive inhibitor, whereas $VBP_{\text{intestine}}$ was a mixed inhibitor (Fig. 2). The inhibition constants for the inhibitor binding with free enzyme, K_i , and with enzyme-substrate complex, K'_i were obtained from the secondary replots of Lineweaver-Burk plots. K_i of $VBP_{\text{blood plasma}}$ and $VBP_{\text{intestine}}$ were 2.57 and 3.49 $\mu\text{g/ml}$, respectively. Kinetic parameters, K_m , V_{max} , k_{cat} , and k_{cat}/K_m of the native enzyme and the enzyme inhibited by the two VBPs were also calculated. Compared with the native enzyme, the apparent K_m values ($K_{m,\text{app}}$) of the inhibited enzyme by $VBP_{\text{blood plasma}}$ and $VBP_{\text{intestine}}$ at 8 $\mu\text{g/ml}$ increased from 1.39 to 7.63 and 4.29 mM, respectively (supplementary material). K_m is the substrate concentration required to produce a rate of $V_{\text{max}}/2$. Therefore, $VBP_{\text{blood plasma}}$ was

Table 2 Antioxidant activity of the vanadium binding proteins of sea squirt *H. roretzi*

Compound	IC_{50}/EC_{50}^* ($\mu\text{g/ml}$)			
	DPPH	ABTS	H_2O_2	FRAP
$VBP_{\text{intestine}}$ (crude)	314.0 ± 0.71^b	645.2 ± 1.08^b	NI**	552.8 ± 0.70^a
$VBP_{\text{intestine}}$ (purified)	119.3 ± 0.70^d	360.4 ± 4.19^d	230.7 ± 4.37^b	146.8 ± 0.71^d
$VBP_{\text{blood plasma}}$ (crude)	367.4 ± 0.58^a	769.4 ± 4.05^a	NI	447.4 ± 0.82^b
$VBP_{\text{blood plasma}}$ (purified)	177.3 ± 0.87^c	427.2 ± 6.24^c	301.8 ± 5.00^a	210.8 ± 4.89^c
Ascorbic acid	41.8 ± 0.68^e	64.2 ± 0.06^e	83.8 ± 0.61^c	127.0 ± 0.16^e

* EC_{50} value was for the FRAP assay

**No inhibition at 1 mg/ml

^{a-e}Mean values with different superscripts in the same column are significantly different ($p < 0.05$)

Table 3 α -Glucosidase inhibitory activity of the vanadium binding proteins of sea squirt *H. roretzi*

Compound	IC ₅₀ (μ g/ml)			
	<i>S. cerevisiae</i> α -glucosidase	<i>B. stearothermophilus</i> α -glucosidase	Sucrase	Maltase
VBP _{blood plasma}	12.6 $\pm$ 0.12 ^c	8.6 $\pm$ 0.06 ^b	NI*	1233.9 $\pm$ 28.84 ^b
VBP _{intestine}	28.3 $\pm$ 0.18 ^b	12.0 $\pm$ 0.13 ^a	NI	1673.2 $\pm$ 31.79 ^a
Ammonium metavanadate	NI	NI	NI	NI
Acarbose	1625.1 $\pm$ 4.02 ^a	0.1 $\pm$ 0.00 ^c	11.1 $\pm$ 0.09	986.6 $\pm$ 45.64 ^c

*No inhibition at 1 mg/ml

^{abc}Mean values with different superscripts in the same column are significantly different ($p < 0.05$) **Fig. 2** Lineweaver-Burk plots of **a** VBP_{blood plasma} and **b** VBP_{intestine} of the sea squirt against *S. cerevisiae* α -glucosidase at different concentrations of *p*NPG, and replots of **c** VBP_{blood plasma} and **d** VBP_{intestine}. Mean values are significantly different ($p < 0.05$)

more effective than VBP_{intestine} due to higher K_m value. The specificity constant (k_{cat}/K_m) indicates the catalytic efficiency of the conversion of *p*NPG to *p*-nitrophenol by *S. cerevisiae*. k_{cat}/K_m of the native enzyme was 7.49×10^3 (M s)⁻¹, whereas the inhibited enzyme by VBP_{blood plasma} and VBP_{intestine} were 1.36×10^3 and 1.53×10^3 (M s)⁻¹, respectively. Therefore, the catalytic efficiency decreased more effectively by VBP_{blood plasma} than VBP_{intestine}.

Combined effect of the two inhibitors on rat intestinal maltase

Rat intestinal α -glucosidase was inhibited by the two combined VBPs better than the single VBP. It was of interest to establish whether the two VBPs interact synergistically to inhibit rat intestinal α -glucosidase or not. Hence, the assay was performed using VBP_{blood plasma} alone and the combined with VBP_{intestine}. Rat intestinal α -glucosidase inhibitory activity of VBP_{blood plasma} at 500 μ g/ml was lower than that of acarbose at the same concentration (Fig. 3). When it was combined with VBP_{intestine} at

Fig. 3 The combined effect of VBP_{blood plasma} and VBP_{intestine} on the inhibition of rat intestinal maltase. (1) 500 µg/ml acarbose; (2) 500 µg/ml VBP_{blood plasma}; (3) 500 µg/ml VBP_{blood plasma} + 100 µg/ml VBP_{intestine}; (4) 500 µg/ml VBP_{blood plasma} + 125 µg/ml

VBP_{intestine}; (5) 500 µg/ml VBP_{blood plasma} + 200 µg/ml VBP_{intestine}; (6) 500 µg/ml VBP_{blood plasma} + 250 µg/ml VBP_{intestine}. The value of the combination index (CI) is indicated below the respective sample. Mean values are significantly different ($p < 0.05$)

low concentrations, the inhibitory activity markedly increased compared to those of acarbose and VBP_{blood plasma} at 500 µg/ml. When combination index (CI) is equal to 1, the two inhibitors do not react with each other. If it is higher than 1, it is considered as an antagonistic interaction, but below than 1, the combination is synergistic (Martinez-Irujo et al. 1998). The CI of the two combined inhibitors at the fixed concentration of VBP_{blood plasma} and the increased concentrations of VBP_{intestine} from 100 to 250 µg/ml were 0.48, 0.46, 0.38 and 0.37, respectively. Therefore, there was a synergistic interaction of VBP_{blood plasma} and VBP_{intestine} on the α -glucosidase inhibitory activity.

Conclusion

Two vanadium binding proteins with strong α -glucosidase inhibitory activity were purified from the intestine and blood plasma of the sea squirt. The VBP of blood plasma was a competitive inhibitor, while that of intestine did a mixed type inhibition against *S. cerevisiae* α -glucosidase. The combined two VBPs inhibited rat intestinal α -glucosidase synergistically. Although the two VBPs didn't have potent antioxidant activities compared to the positive control, the purified compounds increased the antioxidant activity greatly compared with the crude extracts. Therefore, VBPs of the sea squirt have the potential to be developed as a novel nutraceutical in the management of diabetes because of its strong α -glucosidase inhibitory activity, as well as to utilize the processing by-products, intestine and blood plasma of the sea squirt.

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