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# cDNA cloning and characterization of vanadium-dependent bromoperoxidases from the red alga *Laurencia nipponica*

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**The marine red alga genus *Laurencia* is one of the richest producers of unique brominated compounds in the marine environment. The cDNAs for two *Laurencia nipponica* vanadium-dependent bromoperoxidases (*Ln*VBPO1 and *Ln*VBPO2) were cloned and expressed in *Escherichia coli*. Enzyme assays of recombinant *Ln*VBPO1 and *Ln*VBPO2 using monochlorodimedone revealed that they were thermolabile but their $K_m$ values for $Br^-$ were significantly lower than other red algal VBPOs. The bromination reaction was also assessed using laurediol, the predicted natural precursor of the brominated ether laurencin. Laurediol, protected by trimethylsilyl at the enyne, was converted to deacetyllaurencin by the *Ln*VBPOs, which was confirmed by tandem mass spectrometry. Native *Ln*VBPO partially purified from algal bodies was active, suggesting that *Ln*VBPO is functional *in vivo*. These results contributed to our knowledge of the biosynthesis of *Laurencia* brominated metabolites.**

**Key words:** vanadium-dependent bromoperoxidase; *Laurencia*; laurencin; acetogenins; biosynthesis

Since the pioneering work on the isolation of the brominated compound laurencin (**1**) from the red alga *Laurencia nipponica*,[1,2] many halogenated compounds, mostly brominated compounds, have been reported from algae. To date, more than 600 metabolites have been identified from *Laurencia* spp. with several diverse and unique carbon skeletons, including $C_{15}$ acetogenins, sesquiterpenoids, diterpenoids, and triterpenoids.[3] It is known that some of these compounds show cytotoxicity and antibacterial activitiy.[3] Hence, *Laurencia* metabolites are potentially useful for pharmaceutical applications. Despite the increasing number of reports of halogenated compounds isolated from *Laurencia* spp., studies on the biosynthesis of these halogenated compounds are limited.

The leading work focused on the bromination step of the $C_{15}$ acetogenins of *Laurencia* spp., in particular **1**. These early studies achieved the enzymatic bromination of the deduced precursor of **1**, laurediol (**2**),[4] using commercially available lactoperoxidase (LPO) or partially purified bromoperoxidase (BPO) from *L. nipponica*.[5,6] These first studies also achieved the enzymatic synthesis of a final precursor of **1**, deacetyllaurencin (**3**) (Scheme 1). Although the yields of this synthesis were low (1 mg from 73.8 mg using LPO, 0.02 mg from 101 mg using BPO), the studies suggested that LPO- or BPO-like activities could catalyze the bromination of *Laurencia* metabolites.

BPO from *L. nipponica* has been only partially purified,[6] and its structural and biochemical information have remained largely unknown. On the other hand, BPOs have been isolated from other algae, and most of these BPOs have been identified as vanadium-dependent bromoperoxidases (VBPOs) that require vanadate ($VO_4^{3-}$) as cofactor.[7–14]

Therefore, we cloned and expressed VBPOs from *L. nipponica* to clarify the bromination step of secondary metabolites in *Laurencia* spp. Evaluation of the activities of the VBPOs was conducted using a surrogate compound and a physiologically relevant precursor.

## Materials and methods

*RNA preparation.* *L. nipponica* Yamada was collected in December 2009 at Oshoro-bay (Hokkaido, Japan) [15] and was kindly shared by Dr T. Abe (The Hokkaido University Museum). The algal bodies were stored at −80 °C until use. Total RNA was extracted with the RNeasy Midi kit (Qiagen, Venlo, Netherlands) followed by disruption of algal bodies (1 g) into powder in liquid nitrogen. Poly $(A)^+$ RNA was purified using the Oligotex™-dT30 < Super > mRNA purification kit (Takara, Shiga, Japan).

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*Abbreviations*: *Ln*VBPOs, vanadium-dependent bromoperoxidases from *Laurencia nipponica*; MCD, monochlorodimedone; TMS, trimethylsilyl; RACE PCR, rapid amplification of cDNA ends; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2ME, 2-mercaptoethanol; CBB, coomassie brilliant blue; VHPO, vanadium-dependent haloperoxidase; VCPO, vanadium-dependent chloroperoxidase; VIPO, vanadium-dependent iodoperoxidase.

*cDNA cloning of LnVBPOs.* Double stranded cDNA (dscDNA) was synthesized from Poly $(A)^+$ RNA with the Superscript™ Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen, Carlsbad, CA, USA). For rapid amplification of cDNA ends (RACE PCR), adaptor sequences were attached to dscDNA with the Marathon cDNA amplification kit (Takara). The primer pair for 5′-RACE was AP1 (Marathon kit) and Rv1 (5′-GCCGGCATCAAAGAACGCCTTGAG-3′). For 3′-RACE, the primer pair was AP1 and Fw1 (5′-CAGTAGTGGACAGTGACTTGC-3′). Fw1 and Rv1 were designed by referring to the VBPO cDNA sequences of the red alga *Porphyra yezoensis* (the accession number AU187606, AU189808, and AU196993),[16] which were retrieved from the open EST database of Kazusa DNA Research Institution (Chiba, Japan). Takara Ex *Taq*. (Takara) was utilized for RACE PCR under the following conditions: 94 °C for 3 min, 28 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, followed by a final elongation at 72 °C for 3 min. After verifying the presence of the PCR products by 1.0% TBE agarose gel electrophoresis, PCR products were excised with the QIAquick Gel Extraction kit (Qiagen) and subcloned into the pGEM-T Easy Vector (Promega, Fitchburg, WI, USA). Plasmids were transformed into DH5α *E. coli* cells and purified with the QIAprep Spin Miniprep kit (Qiagen) for nucleotide sequencing. Finally, two cDNA sequences encoding VBPO-like proteins, *Ln*VBPO1 (AB830711) and *Ln*VBPO2 (AB847108), were obtained. The cDNA sequences were subjected to alignment and phylogenic analysis using BioEdit [17] and FigTree (http://tree.bio.ed.ac.uk/software). Monomer structure models of *Ln*VBPOs were predicted by SWISS-MODEL.[18–20] Predicted models were visualized with PyMOL (http://www.pymol.org/).

*Construction of gene expression plasmids.* DNA inserts were prepared by PCR using primer sets with a *Nde*I site on the start codon and a *Bam*HI site after the stop codon, respectively (*Ln*VBPO1_Ex_Fw: 5′-CATACA*CATATG*GACCACTACACTCACGTT-3′ and *Ln*VBPO1_Ex_Rv: 5′-TTAACATTG*GGATCC*ATATCTAATTCGAAT-3′, *Ln*VBPO2_Ex_Fw: 5′-CCCACC*CATATG*GAACACTACACTCACATC-3′ and *Ln*VBPO2_Ex_Rv: 5′-AGCCTACAT*GGATCC*CTACAAGTCACGGC-3′). KOD polymerase (Toyobo, Osaka, Japan) was utilized for PCR amplification under the following conditions: 94 °C for 2 min followed by 30 cycles at 98 °C for 10 s, 56 °C for 30 s, and 68 °C for 2 min. The PCR products were excised from a 1.0% TBE agarose gel and digested with *Nde*I and *Bam*HI. The digested products were then introduced into the corresponding sites of pET21a or pET15b vectors (Merck, Darmstadt, Germany). pET21a was used for expression of the untagged *Ln*VBPOs (*Ln*VBPOs) and the C-terminal 6 × His tagged (*Ln*VBPOs-C) proteins. pET15b was used for the N-terminal 6 × His tagged (*Ln*VBPOs-N) proteins. Expression plasmids of *Ln*VBPOs-C were constructed from the plasmids of untagged *Ln*VBPOs by conversion the 3′-terminal regions as follows. First, PCR amplification of the 3′-terminal region was conducted using a primer set designed to amplify upstream of the *Sal*I site of *Ln*VBPO1 (*Ln*VBPO1_Fw2: 5′-AACACACAGATGGACCGTGATA-3′) or the *Cla*I site of *Ln*VBPO2 (*Ln*VBPO2_Fw2: 5′-CACCGTCGCCGGGGCGTGCACAA-3′), and the 3′-terminal region of *Ln*VBPOs. The 3′-terminal primers were designed to replace the stop codon with *Xho*I (*Ln*VBPO1_3t: 5′-ACG*CTCGAG*CACCGTCACGCAACTGC-3′ and *Ln*VBPO2_3t: 5′-AAA*CTCGAG*CGCACGTGCCATCAAAC-3′) and amplified using Takara Ex *Taq* polymerase as described above. The PCR products were digested with *Sal*I and *Xho*I or *Cla*I and *Xho*I and were then used to replace the corresponding regions of the original *Ln*VBPO constructs. All expression plasmids were electroporated into the *E. coli* expression strain BL21 (DE3) pLysS (Takara).

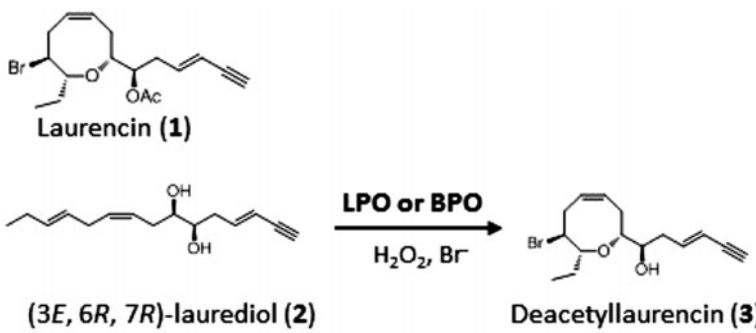
*Purification of recombinant* Ln*VBPO proteins.* Heterologous expression of *Ln*VBPO1 and *Ln*VBPO2 was conducted similarly to the experiments conducted with *Corallina pilulifera* and *C. officinalis*.[10,11,21] Overnight cultures of *E. coli* BL21 (DE3) pLysS were inoculated in 1 L of Luria–Bertani (LB) medium (1%) containing 50 μg/mL of carbenicillin disodium salt and incubated until mid log phase at 37 °C with shaking (120 rpm). The expression of recombinant protein was induced by adding 0.4 mM IPTG at 25 °C. After 3 h, the bacterial cells were harvested by centrifugation (5,000 × *g*, 15 min). The pellet was suspended into the "basic buffer" of this study, consisting of 50 mM Tris-$SO_4$ (pH 8.0) and 1 mM $NH_4VO_3$, and the cells were disrupted with a sonicator. After exclusion of cell debris by centrifugation (15,000 × *g*, 15 min), the obtained supernatant was subjected to 30% saturation of ammonium sulfate for precipitation, while stirring overnight at 4 °C. After ammonium sulfate precipitation, centrifugation (9,000 × *g*, 30 min) was performed to recover the supernatant. The supernatant was dialyzed against the basic buffer and then subjected to DE52 anion exchange chromatography (GE Healthcare, Buckinghamshire, UK). A series of KBr solutions (0.1, 0.2, 0.3, 0.4, and 0.8 M) in the basic buffer were prepared for stepwise elution. After washing the column with the basic buffer, stepwise elution was started. The eluted fractions were monitored by the absorbance at 280 nm and checked by 10% SDS-PAGE under reducing conditions (2-mercaptoethanol, 2ME+) according to Laemmli.[22] *Ln*VBPO1 and *Ln*VBPO2 were eluted with 0.4 and 0.8 M KBr, respectively. Peak fractions of *Ln*VBPO1 and *Ln*VBPO2 were concentrated after dialysis against the basic buffer. Protein concentrations were quantified by the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Purified *Ln*VBPO proteins were stored at 4 °C. The 6 × His tagged recombinant proteins of *Ln*VBPOs were prepared similarly until the recovery of cell lysates. The soluble fraction after the sonication step was subjected to Ni-NTA purification (Invitrogen).

*Purification of algal BPO native protein and peptide sequence analysis.* Algal bodies of *L. nipponica* Yamada were collected in July 2011 at Oshoro-bay

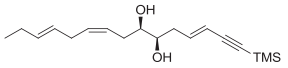
(Hokkaido, Japan).[15] After washing with autoclaved seawater, algal bodies were immediately stored at −80 °C until use. Algal bodies (wet weight of 185 g) were homogenized with a mixer in 600 mL of 50 mM Tris-$SO_4$ (pH 8.0). Homogenized samples were filtered through cheese cloth and centrifuged (6,000 × *g*, 20 min). The supernatant was subjected to purification of native BPO. The method used for recombinant *Ln*VBPO purification was also used for native BPO purification. Proteins with the highest BPO activities eluted in the 0.3 and 0.4 M KBr fractions of DE52 and were subsequently dialyzed against the basic buffer. Partially purified BPO was analyzed by 10% SDS-PAGE (2ME+). The Coomassie Brilliant Blue (CBB)-stained bands were excised from the gel, and trypsin-digested peptide fragments were prepared using the In-Gel Tryptic Digestion Kit (Thermo Scientific). The peptides were desalted using ZipTip C18 filters (Millipore, Billerica, MA, USA) and subjected to nanoLC–MS/MS analysis. EASY-nLC and LTQ Orbitrap Discovery (Thermo Scientific) were used for this experiment. The elution conditions were as follows, using an EASY-Column C18-A2 (10 cm, ID 75 µm) (Thermo Scientific): linear gradients of 5–35% MeCN, 0.1% HCOOH (0–10 min) and 35–100% MeCN, 0.1% HOOH (10–12 min) and an isocratic of 100% MeCN, 0.1% HCOOH (12–20 min), at a flow rate of 300 nL/min. Recorded MS/MS fragments were subjected to the analyzing software, Proteome Discoverer (Thermo Scientific), and MASCOT was used for protein identification.

*Enzyme assay.* BPO activity was assayed spectrophotometrically at 25 °C by measuring the decrease in the absorbance at 290 nm upon bromination of monochlorodimedone (MCD, $\varepsilon = 19.9\ mM^{-1}\ cm^{-1}$, pH 6.0) to monobromo-monochlorodimedone. The standard condition was 50 mM MES-NaOH (pH 6.0), 200 mM KBr, 50 µM MCD, and 1 mM $H_2O_2$.[23] One unit (U) was defined as the conversion of 1 µmol of MCD per minute. MCD was purchased from Alfa Aesar (Ward Hill, MA, USA). The pH dependency was tested by performing the MCD assay at different reaction pH values (pH 4.0 to 6.0 adjusted by 50 mM MES-NaOH, and pH 7.0 to 9.0 adjusted by 50 mM Tris-HCl). The optimum pH was defined as the reaction pH at which the observed activity was the highest. The thermal stability of the *Ln*VBPOs was analyzed using the standard assay conditions, after treatment of the enzymes at various temperatures (25–75 °C) for 20 min, by comparing the activity at each temperature with that of 25 °C. For tolerance to polar organic solvents, each organic solvent was added to the MCD reaction mixture at a final concentration of 40% (v/v). The chlorination activities were detected at 1.5 M KCl with VBPOs from the brown alga *Ascophyllum nodosum* [24] and the red alga *C. pilulifera*.[25] Then, chlorination activities with *Ln*VBPOs were tested at 1.5 M,[24,25] 1 M, 100 and 10 mM KCl (99.5% purity of KCl) instead of KBr. In addition, chlorination was detected at pH 4.5 for the VBPO from *C. officinalis*,[26] the assays at each concentration of KCl were conducted at pH 6.0 and 4.5.[26] Steady-state kinetic experiments were operated by varying the concentration of one of the substrates (0.01, 0.03, 0.06, 0.1, and 1 mM $H_2O_2$ or 0.1, 0.25, 0.5, 1, and 2.5 mM KBr), while fixing the concentration of the second substrate at a saturating concentration (200 mM KBr to determine the $K_m$ for $H_2O_2$, 1 mM $H_2O_2$ to determine the $K_m$ for $Br^-$). Before conducting the kinetic experiment for $Br^-$, the KBr concentration which shows the highest activity at minimum concentration was explored and set at 2.5 mM KBr. The kinetic experiments were performed at pH 6.0 to compare the $K_m$ values to the other red algae. $K_m$ values were derived from non-linear fitting to initial rate data using GraphPad Prism (GraphPad Software, San Diego, CA, USA). *Ln*VBPOs were used for enzyme assays after dialysis against the basic buffer to ensure the coordination of vanadate into the V center.

*Bromination of laurediol.* To assess the ability of recombinant *Ln*VBPOs to brominate the putative precursor of laurencin (**1**), we conducted *in vitro* bromination for putative precursor of **1**, (3*E*, 6*R*, 7*R*)-laurediol (**2**). Fukuzawa et al. reported that (3*E*, 6*R*, 7*R*)-**2** is highly unstable,[5] and 101 mg of **2** added to the enzymatic reaction produces only 0.02 mg of the expected reaction product.[6] Hence, we utilized a relatively stable derivative of (3*E*, 6*R*, 7*R*)-**2**, trimethylsilyl (TMS)-capped (3*E*, 6*R*, 7*R*)-laurediol (**4**). Compound **4** was prepared according to the same procedures utilized by Fukuzawa *et al.*: successive alkaline hydrolysis, silylation, and reduction with Zn metal of **1** (Supplemental Scheme 1).[5]




Scheme 1. Enzymatic bromination and cyclization of *Laurencia* brominated $C_{15}$ acetogenin.
Note: LPO denotes commercially available lactoperoxidase. BPO is bromoperoxidase which was partially purified from *L. nipponica*.[5,6]




TMS-capped (3*E*, 6*R*, 7*R*)-laurediol (4)

The enzymatic reaction was performed under an argon gas atmosphere. Compound **4** (4.8 mg) was dissolved in a small volume of EtOH and added to 63 mM phosphate buffer (pH 6.1) containing 5 mM KBr. Recombinant *Ln*VBPO1 and *Ln*VBPO2 (approximately 100 μg) were added to the mixture to make a final volume of 9 mL. As a negative control, the reaction was also conducted in the absence of the enzyme. The reaction was started by intermittently adding 1 mL of 2 mM $H_2O_2$ for 1 h using a syringe. The reaction was conducted at 25 °C for 24 h in the dark with gentle agitation. After 24 h, the reaction products in the soluble fraction of ethyl acetate were analyzed by LC–MS/MS (LTQ Orbitrap Discovery). YMC Pack Pro C18 (2.0 × 150 mm) (YMC Co., Kyoto, Japan) was selected as the analytical column. The elution condition was a linear gradient of 50–100% MeCN in water (0–20 min) followed by 100% MeCN (20–30 min), at a flow rate of 300 μL/min.

## Results

### *cDNA cloning and expression of LnVBPOs*

Two types of VBPO cDNAs were obtained from *L. nipponica* by RACE PCR. Sequence analysis showed that the cDNAs encoded open reading frames of 643 (*Ln*VBPO1) and 651 (*Ln*VBPO2) amino acids. The

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CpVBPO1   ----MGIPADNLQSRAKASFDTRVAAAELALNRGVVPSFANGEELLYRNPDPDNTDPSFIASFTKGLPHDDNGAI-IDPDDFLAFVRAINSGDEKEIADL
CoVBPO    ----MGIPADNLQSRAKASFDTRVSAAELALARGVVPSLANGEELLYRNPDPENGDPSFIVSFTKGLPHDDNGAI-IDPDDFLAFVRAINSGDEKEIADL
CpVBPO2   ----MGIPADNLQSRAKASFDTRVAAAELALARGVVPSFANGEELLYRN--CETGDPSFIASFTKGLPHDDNGAI-IDPDDFLAFVRAINSGDEKEIADL
LnVBPO1   MDHYTHVQFD--WARRNAAFDVRLRAAVQSRD----ASYS-GDDGHVTNGDEARFPTTLLGTFTKGLPHSLPLALPTDRSAFDAFVSGVNTGDLDTIKSI
LnVBPO2   MEHYTHIPFD--WARRNAAFDVRLRTAVQSRE----ASYS-GDDGHVTNGDEARFPTTLLGTFTKGLPHSLPLALPTDRSAFDAFVSGVNTGDLDTIKSI

CpVBPO1   TLGPARDPETGLPIWRSDLANSLELEVRGWENSSAGLTFDLEGPDAQSIAMPPAPVLTSPELVAEIAELYLMALGREIEFSEFDSP-----KNAEYIQFA
CoVBPO    TLGPARDPDTGLPIWRSDLANSLELEVRGWENSSAGLTFDLEGPDAQSIAMPPAPVLTSPELIAEIAELYLMALGREIEFSEFDSP-----KNAEYIQFA
CpVBPO2   TLGPARDPETGLPIWRSDLANSLELEVRGWENSSAGLTFDLEGPDAQSVAMPPAPVLMSPELIAEMAELYLMALGRDIEFSEFESP-----KNAAFIRSA
LnVBPO1   PLGPDPEGDNSF----SSSAMTTGAKVRGWESMAAGLAFTLEAPDPQRVTMPPAPQIDSVELGYELSESYWMALCRDIAFTEFDNSDTVAEAAASMAAHA
LnVBPO2   PLGPDPEGDNSF----SSSAMTTGAKVRGWESMAAGLAFTLEAPDPQRVTMPPAPQIDSVELGYELSESYWMALCRDIAFTEFDNSDTVAEAAASMAAHA

CpVBPO1   IDQLNGLEWFNTPAKLGDPPAEIRRRRGE-VTVGNLFRGILPGSEVGPYLSQYIIVGSKQIGSATVGNKTLVSPNAADEFDGEIAYGSITISQRVRIATP
CoVBPO    IDQLNGLEWFNTPAMLGDPPAEIRRRRGE-VTVGNLFRGILPGSEVGPYLSQYIIVGSKQIGSATGGNKTLVSPNAADEFDGEIAYGSITISQRVRIATP
CpVBPO2   IERLNGLEWFNTPAKLGDPPAEIRRRRGE-VTVGNLFRGILPGSEVGPYLSQYIIVGSKQIGSATVGNKTFVSPNAADEFDGEIAYGSITISQRVRIATP
LnVBPO1   WVAEDGCDQLGELTPTQTAHRELEMKNGEGITQGTIFRGSLNGDNIGPYLSQFLLIGCDTLGSR------FSKDDPESYLTGHIRFGAHRIDQRVRKAVP
LnVBPO2   WVAEDGCDQLGELTPTQTAHRELEMKNGEGITQGTIFRGSLNGDNIGPYLSQFLLIGCDTLGSR------FSNDDPESYLTGHIRFGAHRIDQRVRKAVP

CpVBPO1   GRDFMTDLKVFLDVQDAADFRGFESYEPG----------ARLIRTIRDLATWVHFDALYEAYLNACLILLANGVPFDPNLPFQQEDKLDNQDVFVNFGSA
CoVBPO    GRDFMTDLKVFLDVQDAADFRGFESYEPG----------ARLIRTIRDLATWVHFDALYEAYLNACLILLANRVPFDPNIPFQQEDKLDNQDVFVNFGDA
CpVBPO2   GRDFMTDLKVFLDVQDGADFRGFESYEPG----------ARLIRTIRDLATWVHFDALYEAYLNACLILLANGVPFDPNLPFQQEDKLDNHDVFVNFGSA
LnVBPO1   DRDYMTTWGAYIDVANGADVRGRESYVTAEGDNFNDEETHRFIHTPRDLATYVHYDALYQPYINACLLMLAMGIEYDAGLPFGDEDEVDKQTGFALFGGP
LnVBPO2   DRDYMTTWGAYIDVANGADVRGRESYVTAEGDNFNDEETHRFIHTPRDLATYVHYDALYQPYFNACLLMLAMGIGYDEGLPFGDEDEVDKQTGFALFGGP

                          *      *
CpVBPO1   HVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISVNKIA----PQKG---ESIFPEVDLAVEELG-DILEKAEISNRKQN----------------
CoVBPO    HVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISVNKIA----AEKG---ESVFPEVDLAVEELE-DILEKAEISNRKQN----------------
CpVBPO2   HVLSLVTEVATRALKAVRYQKFNIHRRLRPEATGGLISVNKKS----FLAGS--DIIFPEVSELVEELS-SILDDVAESNEKQN----------------
LnVBPO1   HILSLLAEVVLKALKAVRFQKFAMHRRARPETVAGWIDCVQRPRTSDEQEGNEGQTVFKPIEKLCQYVDSDLLQKVREHNVTQNNTQMDRDIARLYDYDP
LnVBPO2   HILSLLAEVVLKALKAVRFQKFAMHRRARPETVAGWIDCVQRPRTSDEQEGNEGQTVFKPIEKLCQYVDSDLLQKVREHNVTQNNTQMDRDITRLHDYDP

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CpVBPO1   ---IADGDPDPDP----------------------SFLLPMAFAEGSPFHPSYGSGHAVVAGACVTILKAFFDSGIEIDQVFEVDKDEDKLVKSSFKGTL
CoVBPO    ---IADGDPDPDP----------------------SFLLPQAFAEGSPFHPSYGSGHAVVAGACVTILKAFFDSNFQIDQVFEVDKDEDKLVKSSFKGTL
CpVBPO2   ---RADGIVSPDK----------------------SFLLPMAFAEGSPFHPSYGSGHAVVAGACVTILKAFFDANFQIDKVFEVDTDEDKLVKSSFKGTL
LnVBPO1   VDTVADCSAEQQE-----EQTEDEG---QQQQQGGTYLLPMAYAEGSPMHPAYGAGHATVAGACTTILKAFFDTTQTLPYAFTSTEDGTKLTQIEVDEPL
LnVBPO2   VDAVADCAVDQQQQVYEQQQTEDEQNEQQQQQRGGTYLLPMAYAEGSPMHPAYGAGHATVAGACTTILKAFFDTTQKLPYVFTSTDDGTKLVQIDVDEQL

                          *      *
CpVBPO1   TVAGELNKLADNIAIGRNMAGVHYFSDQFESLLLGEQVAIGILEEQSLTYGENFFFNLPKFDGTTIQI   (598 aa)
CoVBPO    TVAGELNKLADNIAIGRNMAGVHYFSDQFESILLGEQVAIGILEEQSLTYGENFFFNLPKFDGTTIQI   (598 aa)
CpVBPO2   TVAGELNKLADNVAIGRNMAGVHYFSDQFESLLLGEQIAIGILEEQSLTYGENFFFNLPKFDGTTIQI   (597 aa)
LnVBPO1   TVEGELNKLCSNISIGRNWAGVHWKSDYTESIKLGEEIAIGLLREQKNVYSEDFSMTIPLFDGSCVTV   (643 aa)
LnVBPO2   TVEGELNKLCSNISIGRNWAGVHWKSDYTESIKLGEEIAIGLLREQKNVYTECFSMTIPLFDGTCVTI   (651 aa)
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Fig. 1. Alignment of the amino acid sequences of red algal VBPOs from *L. nipponica* (*Ln*VBPO1 and *Ln*VBPO2), *C. pilulifera* (*Cp*VBPO1 and *Cp*VBPO2), and *C. officinalis* (*Co*VBPO).

Notes. Alignment data were obtained using CLUSTAL W and displayed by BioEdit.[17] Asterisks (*) indicate the residues that constitute the V center. (#) Denotes the VBPO specific His residue. The arrow shows substitution of Ser to Ala in *Ln*VBPOs (Ala530 and Ala538 in *Ln*VBPO1 and *Ln*VBPO2). The fragment sequences acquired from the algal BPO using nanoLC–MS/MS are underlined (Table 1). The dashed line is the *Ln*VBPOs specific Gln rich insertion. Accession No. of VBPO from each *Corallina* spp. is BAA31261 for *Cp*VBPO1, BAA31262 for *Cp*VBPO2, and AAM46061 for *Co*VBPO. Accession Nos. for *Ln*VBPO1 and *Ln*VBPO2 are AB830711 and AB847108, respectively.

amino acid sequence identity of these two enzymes was 95%. According to sequence alignment analysis, *Ln*VBPO1 and *Ln*VBPO2 contained most of the amino acid residues essential for the V center of vanadium-dependent haloperoxidase (VHPO), including the His residue that covalently binds to vanadate and other residues responsible for hydrogen bonding in the active site. In addition, conserved VBPO-specific His residues were identified (Fig. 1).[7,8]

The recombinant *Ln*VBPOs without tags were purified by ammonium sulfate precipitation and anion exchange chromatography. By SDS-PAGE under reducing conditions (2ME+), the purified proteins migrated as single bands at approximately 77 kDa (Fig. 2). This apparent mass is slightly greater than the theoretical molecular mass of *Ln*VBPO1 (71.02 kDa) and *Ln*VBPO2 (71.12 kDa).

Recombinant *Ln*VBPO1 and *Ln*VBPO2 had high bromination activities of 265 and 275 U/mg protein for MCD, respectively (Table 2). When the recombinant proteins carrying 6 × His tags were assayed under the same conditions, these enzymes exhibited significantly reduced bromination activities; 0.7% (*Ln*VBPO1-N), 0.3% (*Ln*VBPO2-N), 0.1% (*Ln*VBPO1-C), and 0.3% (*Ln*VBPO2-C) of the activities of the corresponding untagged *Ln*VBPOs. Therefore, the untagged *Ln*VBPO proteins were used for further experiments.

*Purification of BPO from* L. nipponica

BPO was partially purified from algal bodies of *L. nipponica*. The partially purified enzyme also migrated around 77 kDa (72 kDa) on a 10% SDS-PAGE reducing (2ME+) gel, and additional bands were observed near 18 kDa (Fig. 3). The partially purified algal BPO (72 kDa) had slightly lower molecular mass than those of recombinant *Ln*VBPOs (77 kDa). This difference of 5 kDa might be caused by the effects of the existence

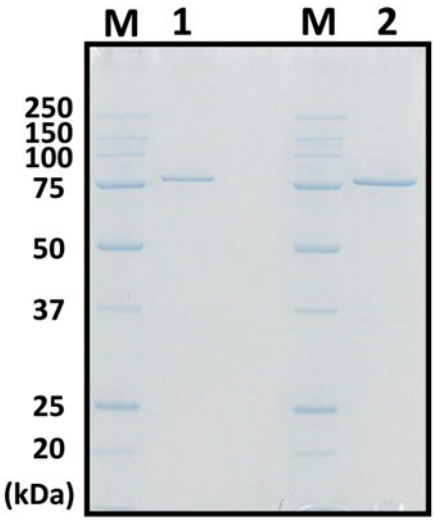


Fig. 2. SDS-PAGE (10%) under reducing conditions (2ME+) of purified *Ln*VBPO1 and *Ln*VBPO2.

Notes: The marker (M) used was the precision plus protein standards (Bio-Rad, Hercules, CA, USA). The lanes are labeled 1 and 2 to denote purified *Ln*VBPO1 and *Ln*VBPO2, respectively. The purified protein migrated to approximately 77 kDa.

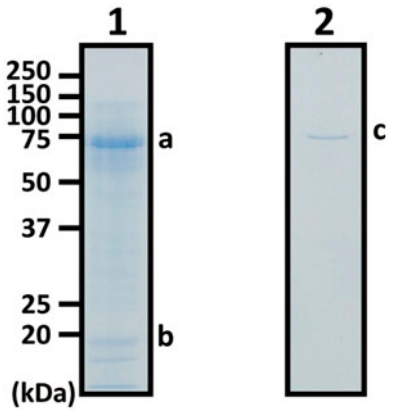


Fig. 3. Comparison of partially purified BPO from algal bodies and recombinant *Ln*VBPO1.

Notes: SDS-PAGE was conducted on a 10% gel (2ME+). Lane 1 is 0.3 M KBr pooled and concentrated MCD-active fractions from DE52 anion exchange chromatography of algal body extracts. Lane 2 is purified recombinant *Ln*VBPO1. The 72 kDa band (a), 18 kDa band (b), and c band were excised from the gel and subjected to peptide sequencing using nanoLC–MS/MS (Table 1).

of other protein(s) around 72–77 kDa or degradation during the concentration of algal BPO. By peptide fragment analysis using nanoLC–MS/MS, the 77 kDa protein (c in Fig. 3) was indeed identified as *Ln*VBPO1 (22.4% coverage). The protein at 72 kDa (a in Fig. 3) purified from algal bodies contained partial sequences that were identical to *Ln*VBPOs (6.22% sequence coverage) (Fig. 1). The other band at 18 kDa (b in Fig. 3) was identified as R-phycoerythrin α chain (15.85% coverage) (Table 1). The lower sequence coverage of partially purified algal BPO (6.22%) compared to recombinant *Ln*VBPO1 (22.4%) might be due to subtypes of BPO or other unknown proteins in the 72 kDa band.

The bromination activity of purified algal BPO was estimated to 33 U/mg protein for MCD, which was lower than the activity of recombinant *Ln*VBPOs (265 in *Ln*VBPO1 and 275 U/mg protein in *Ln*VBPO2) (Table 2). Another difference compared with recombinant *Ln*VBPOs was reduced stability at 4 °C. The partially purified BPO was rapidly inactivated within 1 week.

*Bromination activity of LnVBPOs*

The bromination activity of the recombinant *Ln*VBPOs was evaluated using MCD under different conditions. In the pH-rate profile, the maximum activities of *Ln*VBPO1 and *Ln*VBPO2 were observed at pH 7.0, and the activities were not detected at pH 4.0 (Table 2, Supplemental Fig. 2). The activities of both *Ln*VBPO1 and *Ln*VBPO2 declined when treated at 55 and 65 °C, and were inactive at 65 and 75 °C, respectively (Table 2, Supplemental Fig. 2). When the assay was conducted in the existence of polar organic solvents (40% v/v), *Ln*VBPO1 and *Ln*VBPO2 showed some degree of tolerance except in the presence of acetone (Fig. 4). These experiments indicate that the enzymatic properties of *Ln*VBPO1 and *Ln*VBPO2 are similar, but *Ln*VBPO2 appears to be more stable than

Table 1. List of identified fragment sequences from tryptic digests of a-c in Fig. 3 using nanoLC-MS/MS.

| Sample | Identified protein | $MH^+$ (Da) | $\Delta M$ (ppm) | Expected sequence |
|---|---|---|---|---|
| c (Fig. 3) | *Ln*VBPO1 | 1841.85917 ($Z=3$) | −4.36 | VREHNTVQNNTQMDR |
| | | 1857.85343 ($Z=3$) | −4.67 | VREHNVTQNNTQmDR |
| | | 1183.69768 ($Z=2$) | −5.82 | LGEEIAIGLLR |
| | | 1721.74033 ($Z=3$) | −5.01 | mDHYTHVQFWAR |
| | | 1292.65349 ($Z=2$) | −4.57 | NGEGITQGTIFR |
| | | 1225.67546 ($Z=2$) | −5.95 | FPTTLLGTFTK |
| | | 1486.82975 ($Z=3$) | −5.36 | GLPHSLPLALPTDR |
| | | 1764.84972 ($Z=4$) | −4.13 | FSKDDPESYLTGHIR |
| | | 1602.68534 ($Z=3$) | −4.51 | EHNVTQNNTQmDR |
| | | 1586.68906 ($Z=3$) | −5.41 | EHNVTQNNTQMDR |
| | | 942.43675 ($Z=2$) | −5.03 | SDYTESIK |
| | | 792.39678 ($Z=2$) | −3.97 | NAAFDVR |
| | | 1865.74729 ($Z=3$) | −3.24 | DASYSGDDGHVTNGDEAR |
| | | 661.32079 ($Z=2$) | −4.72 | FAMHR |
| | | 1402.65223 ($Z=3$) | −5.39 | DDPESYLTGHIR |
| | | 770.42717 ($Z=2$) | −4.72 | FIHTPR |
| | | 997.49576 ($Z=3$) | −4.55 | NWAGVHWK |
| | | 948.49620 ($Z=2$) | −5.10 | RNAAFDVR |
| a (Fig. 3) | *Ln*VBPOs | 1486.83057 ($Z=3$) | −4.80 | GLPHSLPLALPTDR |
| | | 1225.67754 ($Z=2$) | −4.25 | FPTTLLGTFTK |
| | | 1764.84755 ($Z=4$) | −4.33 | FSKDDPESYLTGHIR |
| b (Fig. 3) | R-phycoerythrin α chain | 1796.83390 ($Z=3$) | −6.20 | YGYLKNPGEAGENQEK |
| | | 1053.56476 ($Z=3$) | −3.82 | LGSNHEAVVK |

Notes: Data analysis was undertaken using proteome discoverer. The analytical condition was set as oxidation of methionine. $\Delta M$ means the margin in theoretical values. $Z$ in $MH^+$ means detected charge. The m in the expected sequence shows oxidation of a Met residue. The underlined fragments were detected in samples a and c in Fig. 3.

Table 2. Analysis of *Ln*VBPO1 and *Ln*VBPO2.

| | *Ln*VBPO1 | *Ln*VBPO2 |
|---|---|---|
| Bromination activity for MCD | 265 U/mg protein | 275 U/mg protein |
| $K_m$ for $H_2O_2$ at pH 6.0 | 0.026 (±0.006) mM | 0.025 (±0.006) mM |
| $V_{max}$ for $H_2O_2$ | 336 (±20.7) U/mg protein | 343 (±22.2) U/mg protein |
| $K_m$ for $Br^-$ at pH 6.0 | 0.53 (±0.05) mM | 0.35 (±0.07) mM |
| $V_{max}$ for $Br^-$ | 891 (±32.3) U/mg protein | 926 (±55.9) U/mg protein |
| Optimum pH | pH 7.0 | pH 7.0 |
| Inactivation temperature | 65 °C | 75 °C |
| Chlorination activity for MCD | Not detected | Not detected |

Note: $K_m$ for $H_2O_2$ and $Br^-$ were calculated by non linear regression analysis ($N=3$). The pH dependency, the effects of heat treatment (20 min), and the chlorination assay were tested in triplicates.

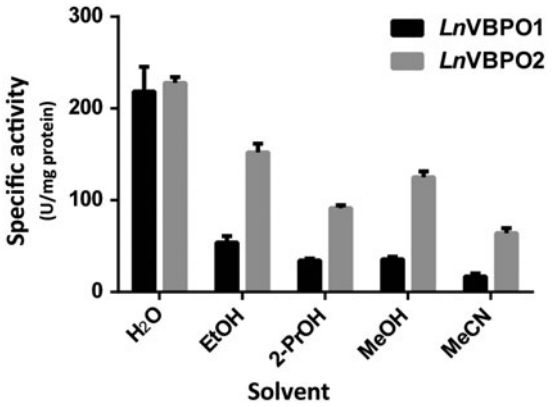


Fig. 4. Tolerance for 40% polar organic solvent ($N=2$).
Notes: In presence of acetone, *Ln*VBPO1 and *Ln*VBPO2 were inactive, and the data for acetone are therefore excluded.

*Ln*VBPO1. The $K_m$ for $H_2O_2$ was 0.026 mM for *Ln*VBPO1 and 0.025 mM for *Ln*VBPO2, and the $K_m$ for $Br^-$ was 0.53 mM for *Ln*VBPO1 and 0.35 mM for *Ln*VBPO2 at pH 6.0 (Table 2). The inhibitory effect of KBr for the MCD bromination activities of *Ln*VBPOs was observed above 50 mM KBr. Chlorination activity was not detected using *Ln*VBPO1 or *Ln*VBPO2 at any KCl concentration tested (10 mM, 100 mM, 1 M, and 1.5 M).

### *Bromination of laurediol*

We synthesized TMS-capped (3*E*, 6*R*, 7*R*)-laurediol (**4**) that is a stable form of (3*E*, 6*R*, 7*R*)-laurediol (**2**), using laurencin (**1**) as a starting material. We then added compound **4** to a small-scale enzyme reaction because compound **2** was highly unstable and it was difficult to prepare a large amount of **2** from the algal bodies.[5] By LC–MS analysis, a

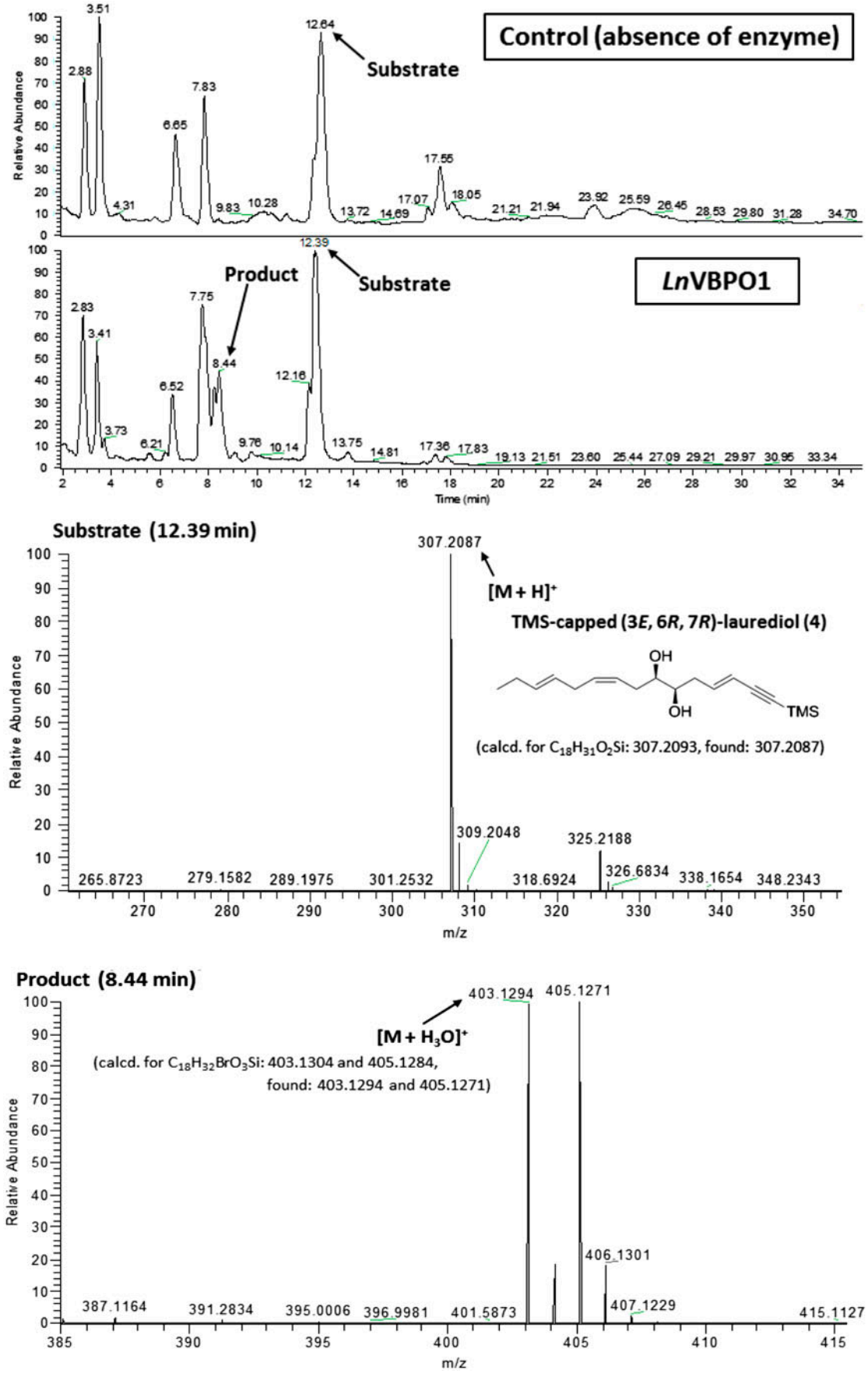


Fig. 5. LC-MS analysis of enzymatic bromination of TMS-capped (3*E*, 6*R*, 7*R*)-laurediol (**4**).

Notes: Unreacted substrate was detected at 12.39 min as $[M + H]^+$: calcd. for $C_{18}H_{31}O_2Si$: 307.2093, found: 307.2087. The hydrate of the expected compound ($[M + H_3O]^+$), *m/z* 403.1294 and 405.1271, was specifically observed at 8.44 min in the samples containing enzyme. Since *Ln*VBPO1 and *Ln*VBPO2 provided the same results, the results are shown only for *Ln*VBPO1. The control is absence of enzyme in the reaction.

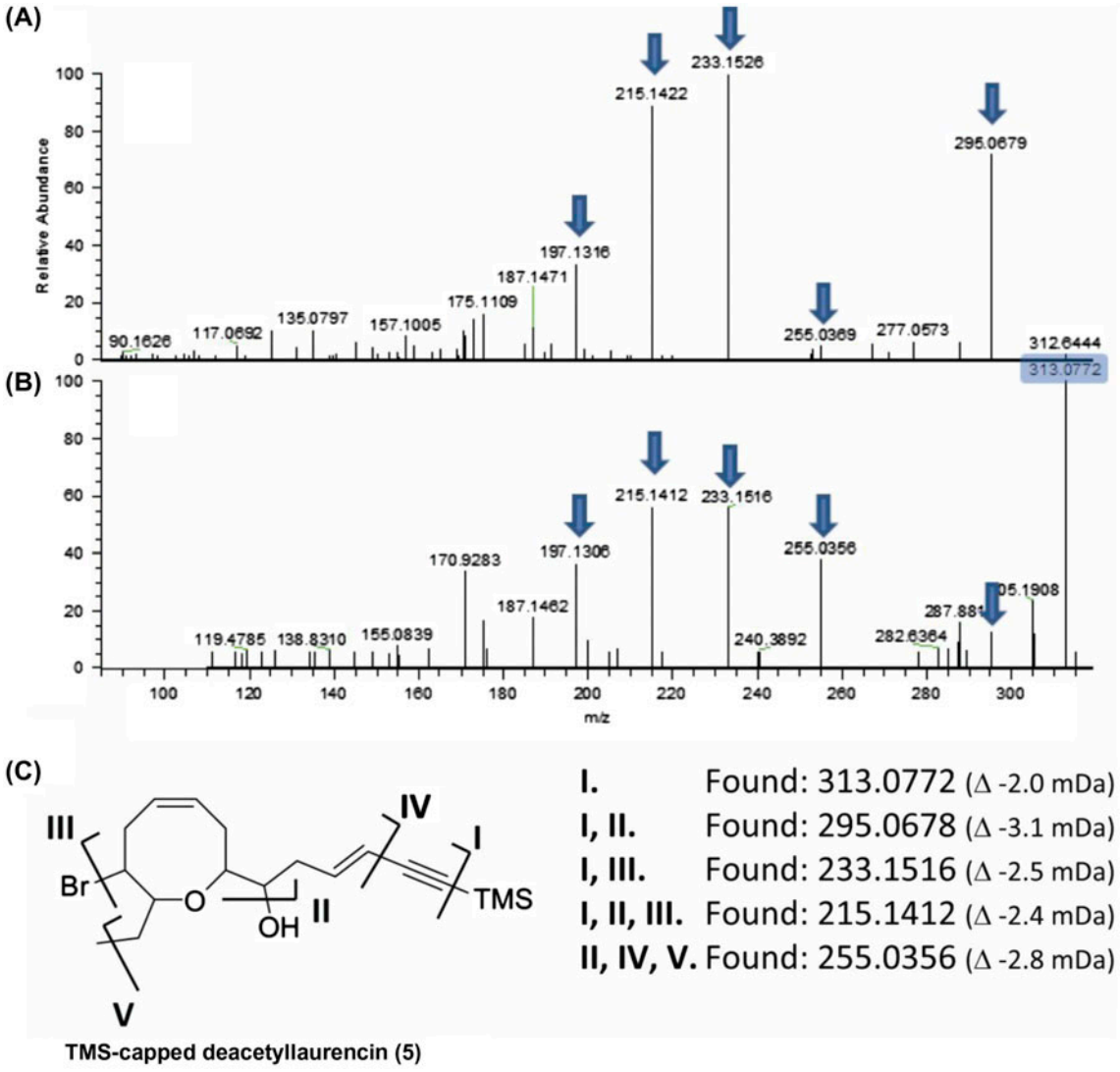


Fig. 6. Comparison of MS/MS fragment patterns of deacetyllaurencin (**3**) and the enzymatic products.

Notes: (A) MS/MS fragment of **3**. (B) MS/MS fragment of *m/z* 403, which was enzymatically produced from **4** (Fig. 5). The fragments were obtained under the same conditions. The spectra of A and B had many identical signals such as *m/z* 295, 255, 233, 215, and 197 (blue arrows). The prediction of the fragmentation was drawn in (C). Symbol Δ denotes the difference between the observed mass and calculated exact mass. Different intensity patterns between (A) and (B) might be caused by the different precursor ions. In B, a signal appeared with the same *m/z* value as the $[M+H]^+$ ion of **3** (313).

mono-brominated compound, which coincided with the hydrate of TMS-capped deacetyllaurencin (**5**) ($[M+H_3O]^+$: calcd. for $C_{18}H_{32}BrO_3Si$: 403.1304 and 405.1284, found 403.1294 and 405.1271), was detected in the reactions of *Ln*VBPO1 and *Ln*VBPO2 (Fig. 5). These mass spectra were not observed in the negative control reaction (in the absence of the enzyme).

The LC–MS/MS fragment pattern of *m/z* 403.1294 was obtained and compared to that of deacetyllaurencin (**3**). Although the MS/MS spectra did not completely match due to the existence of TMS, most of the fragments showed the same pattern as those of **3** such as *m/z* 295.0678 $[M–TMS–OH]^+$ (Δ–3.1 mDa), 255.0356 $[M–TMS–C_2H_2$ (enyne)$–OH–CH_3]^+$ (Δ–2.8 mDa), 233.1516 $[M–TMS–Br]^+$ (Δ–2.5 mDa), 215.1412 $[M–TMS–OH–Br]^+$ (Δ–2.4 mDa), and 197.1306. In particular, the *m/z* of 313.0772 coincided with the $[M+H]^+$ of **3** (Δ–2.0 mDa) and was also present as a fragment of *m/z* 403.1294 (Fig. 6). Upon further fragmentation of *m/z* 313.0772, the fragment pattern was almost identical to that of **3** below *m/z* 305.1901 (data not shown). Therefore, the pair at *m/z* 403.1294 and 405.1271 may correspond to desired product **5**. These results suggest that *Ln*VBPOs catalyzed the bromination and cyclization of (3*E*, 6*R*, 7*R*)-**2** to form **3**.

## Discussion

cDNAs encoding VBPO were cloned from *L. nipponica*. The primary sequences of *Ln*VBPO1 and *Ln*VBPO2 are similar and are predicted to contain the His residue that binds V in the active site (His598 and His606 in *Ln*VBPO1 and *Ln*VBPO2, respectively). The Gln-rich stretches (Gln494–508 in *Ln*VBPO1, Gln494–515 in *Ln*VBPO2) (Fig. 1) are distinct features of the *L. nipponica* haloperoxidases compared with other reported algal VBPOs. In addition, the Ser residue

which hydrogen bonds to the V center of the VBPOs from *A. nodosum*, *C. pilulifera*, and *C. officinalis*,[9,12,13] is replaced by Ala530 and Ala538 in *Ln*VBPO1 and *Ln*VBPO2, respectively (Fig. 1). Phylogenetic analysis showed that the *Ln*VBPOs are branched in a cluster of the red algal VBPOs, such as VBPOs from *C. officinalis* and *C. pilulifera* (approximately 40% identities), and *Gracilaria changii* (46% identity).[27] Notably, VBPOs from the marine cyanobacteria *Synechococcus* spp. CC9311 and WH8020 clustered with red algal VBPOs (Supplemental Fig. 1). This clustering might be the result of horizontal gene transfer between red algae as proposed by Johnson *et al.*[28]

Crystal structures of algal VBPOs indicated that enzymatic activities are achieved by assuming multimeric structures, including a dimer in *A. nodosum* [9] and a dodecamer in *C. officinalis* and *C. pilulifera*.[12,13] The Cys residues that participate in inter- or intra-subunit disulfide bonds in the brown alga *A. nodosum* VBPO [9] are not found in the *Ln*VBPOs. *Ln*VBPOs carrying a 6 × His tag at either the N- or C-terminus significantly reduced the bromination activity in the MCD assay. According to the structural information from other red algal VBPOs from *Corallina* spp., the addition of a 6 × His tag at the N-terminus may disrupt the correct assembly of subunits and consequently impair the enzyme activity.[21] The C-terminus domain adjoins α-helices involved in the formation of a deep cleft near the V center [α19 in *C. officinalis* VBPO [12]]. This implies that the termini regions of the VBPOs have important roles for enzyme activity. The structure/function of *Ln*VBPOs will be investigated by further experiments.

The MCD assay was used to examine the bromination activities of the *Ln*VBPOs. The $K_m$ values for $Br^-$ at pH 6.0 were lower than those reported for native and recombinant VBPOs from other red algae. For instance, in *C. pilulifera*, 0.092 mM for $H_2O_2$ and 11 mM for $Br^-$ assaying native VBPO at pH 6.0,[29] 0.1 mM for $H_2O_2$ and 8.4 mM for $Br^-$ assaying recombinant VBPO at pH 6.5.[30] In *C. officinalis*, 0.06 mM for $H_2O_2$ and 1.0 mM for $Br^-$ assaying native VBPO at pH 6.4,[31] 0.017 mM for $H_2O_2$ and 1.2 mM for $Br^-$ assaying recombinant VBPO at pH 6.5.[11] Most algal VBPOs, native or recombinant proteins, exhibit an optimum pH of approximately 6.0, which is slightly more acidic than the optimum pH of the *Ln*VBPOs (pH 7.0, Table 2). For the VBPO from the red alga *G. changii*, the optimum pH is 7.0, and the difference in optimum pH may depend on the pH in the habitat.[27] The thermal stability of recombinant VBPO from *C. pilulifera* is considerable, and the enzyme activity is maintained even above 80 °C.[25] *Ln*VBPO1 and *Ln*VBPO2 were less stable and inactivated after treatment at 65 and 75 °C when comparing the activity at 25 °C (Table 2).

The *Ln*VBPOs did not show chlorination activities under conditions where chlorination activities were detected in other algal VBPOs (1.5 M KCl at pH 6.0 or 4.5).[24–26] In the fungus *Curvularia inaequalis*, the replacement of Ser402 with Ala of vanadium-dependent chloroperoxidase (VCPO) results in loss of chlorination activity, but retained bromination activity.[32] The conserved Ser residue in algal VBPOs and VCPOs is substituted to Ala530 and Ala538 in *Ln*VBPO1 and *Ln*VBPO2, respectively (Fig. 1). The Ser residue interacts with the V center, likely throughout the catalytic cycle, and is not directly affected by the binding of $H_2O_2$.[33] This Ser has also been proposed to stabilize the protonation state of the peroxo-vanadate intermediate.[33] A Ser-to-Ala substitution has been reported for vanadium-dependent iodoperoxidase (VIPO) from *Laminaria digitata*,[34] VBPOs from *Synechococcus* spp. CC9311 and WH8020,[28] and VBPO from *G. changii*.[27] In VIPO from *L. digitata*, substitution of Ser to Ala may result in the loss of hydrogen bonding and instead recruitment of Lys to the V center to neutralize the negative charge of the vanadate cofactor.[34] Based on the Ser to Ala mutation, it is presumed that the V center of the *Ln*VBPOs is more labile than in VBPOs from *A. nodosum* and *Corallina* spp. (Fig. 7). In *A. nodosum* and *C. pilulifera*, the V center of the VBPOs retains the Ser residue and may thus produce a more active catalytic intermediate than in the *Ln*VBPOs, resulting in chlorination activity under some conditions.[24–26] Moreover, *A. nodosum* VBPO has a Trp residue, which is essential for

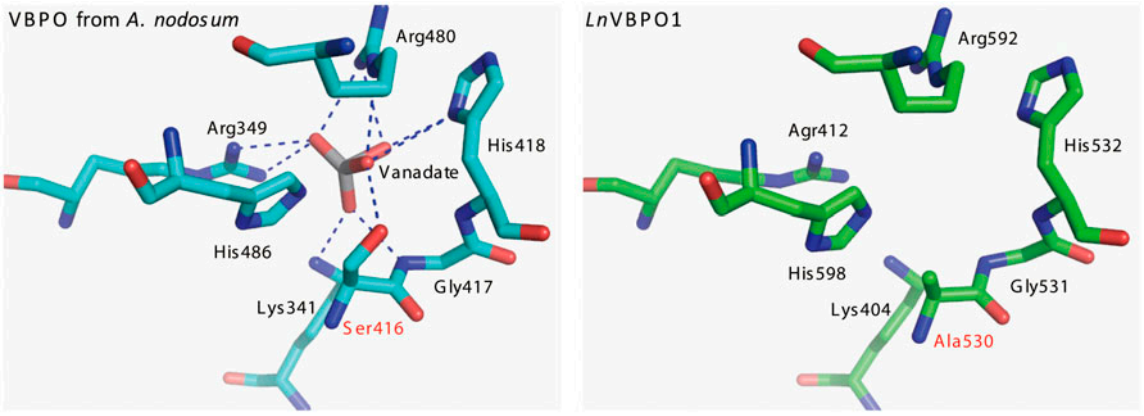


Fig. 7. Comparing the V centers of the VBPOs from *A. nodosum* and the *Ln*VBPOs.

Notes: Structure models of *Ln*VBPOs were predicted by SWISS MODEL[18–20] and the V centers were visualized in PyMOL. As a comparator, VBPO from *A. nodosum* was chosen (PDB accession No. 1QI9), since this enzyme was characterized by crystallography.[9] The amino acid residues essential for the V center are well conserved in the two species, with the exception of the Ser (Ser416 in *A. nosodum*) to Ala (Ala530 in *Ln*VBPO1 and Ala538 in *Ln*VBPO2) replacement shown in red. Since the residues constructing the V center in *Ln*VBPO1 and *Ln*VBPO2 are predicted to be the same, only the model of *Ln*VBPO1 is shown in the figure.

the incorporation of $Cl^-$.[24] The oxidation of chloride, which is more electronegative than bromide, might require a more active peroxo–vanadate complex.[32,33,35]

The same peptide fragments between *Ln*VBPOs and partially purified BPO from algal bodies were verified by nanoLC–MS/MS (Fig. 1 and Table 1), suggesting that *Ln*VBPOs are the functional proteins *in vivo*. The difficulty in separating VBPOs and phycoerythrin has been reported from another red alga *C. officinalis*.[31]

When TMS-capped (3*E*, 6*R*, 7*R*)-laurediol (**4**) was used in the enzymatic bromination reaction, the same MS/MS fragments as those of the desired product, deacetyllaurencin (**3**), were detected (Fig. 6). Three bromination mechanisms that lead to **3** have been proposed: direct ring formation,[5,6] ring-expanding bromoetherification,[36] and intramolecular bromonium ion-associated epoxide ring-opening.[37] Since our bromination experiment was conducted in small scale, further refinement of experimental conditions could lead to structural and kinetic analysis for reaction products to understand the detailed mechanisms of enzymatic bromination reaction of *Laurencia* metabolites.

In summary, we identified cDNAs encoding VBPOs from *L. nipponica* which produces unique brominated compounds in the marine environment.[3] The Gln-rich insertion is a major feature of the *Ln*VBPOs when compared to other algal VBPOs. The bromination of a derivative of the putative precursor of laurencin was verified after the incubation with recombinant *Ln*VBPOs. The *in vitro* enzymatic study contributes to our knowledge about the biosynthesis of unique brominated metabolites from *Laurencia* and builds on previous *in vivo* algal culture experiments.[38]

## Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.918482.

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