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Kouhei Nagai, Koichi Morimoto, Haruka Ikegami, Hajime Kimura, Norishige Yotsukura

Investigation of proteomic profiles of lamina of *Ecklonia kurome* (Laminariales): homology-based cross-species protein identification and analysis of the post-translational processing of vanadium-dependent bromoperoxidases using MALDI-TOF/TOF

Kouhei Nagai, Koichi Morimoto, Haruka Ikegami

Department of Genetic Engineering, Faculty of Biology-Oriented Science and Technology, Kinki University,
Kinokawa, Wakayama 649-6493, Japan

Hajime Kimura

Fisheries Experimental Station, Wakayama Prefecture Research Center of Agriculture, Forestry and Fisheries,
Kushimoto, Wakayama 640-8585, Japan

Norishige Yotsukura

Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido 060-0809, Japan

Author for correspondence: e-mail yotsukur@fsc.hokudai.ac.jp, TEL +81-11-706-2585, Fax +81-11-706-3450

Abstract Proteomic profiles of lamina of *Ecklonia kurome* Okamura, one of the Japanese dominant laminarialean kelps, were investigated by two-dimensional electrophoresis (2-DE) and MALDI-TOF/TOF. Due to the absence of *E. kurome* DNA or protein databases, homology-based cross-species protein identification was performed using a combination of three database-searching algorithms, Mascot peptide mass fingerprinting (PMF), Mascot MS/MS ion search (MIS), and Mass spectrometry based BLAST (MS BLAST). Proteins were extracted from lamina by an ethanol/phenol method, and subjected to 2-DE (pI 4-7, 10% polyacrylamide gel). More than 700 spots were detected in the 2-DE gel with CBB, and 93 spots (24 proteins) were successfully identified by MALDI-TOF/TOF and the cross-species database searching. The identified proteins mainly consisted of cytoplasmic carbohydrate metabolic enzymes, chloroplast proteins involved in photosynthesis, and haloperoxidases. Interestingly, vanadium-dependent bromoperoxidases (vBPO), which is thought to be involved in halogen uptake, synthesis of halogenated products, and detoxification of reactive oxygen species, were separated into at least 23 different spots. By comparing mass spectra, amino-acid sequences predicted from tandem mass spectra, and haloperoxidase activities of the vBPOs, we found that (1) at least two types of vBPOs were expressed in the lamina of *E. kurome*, and (2) two pro-vBPOs might be activated by specific cleavage at N- and C-terminal regions.

Keywords 2-DE / Kelp / MALDI-TOF/TOF / Protein identification/ Haloperoxidase

Abbreviations

2-DE two-dimensional electrophoresis

vBPO vanadium-dependent bromoperoxidase

vHPO vanadium-dependent haloperoxidase

vIPO vanadium-dependent iodoperoxidase

MIS MS/MS ion search

MS BLAST MS spectrometry based BLAST

Introduction

The laminarialean kelps such as *Ecklonia cava* Kjellman, *E. kurome* Okamura and *Eisenia bicyclis* (Kjellman) Setchell are the dominant algal species around the coastline of the main island of Japan and usually form underwater forests. Since the kelp forests contribute substantial primary productivity (Mann 1973; Abdullah and Fredriksen 2004) and provide habitats or food for a wide range of marine organisms, such as many fish and invertebrates including economically important species (Dayton 1985), it is important to reveal their biological characteristics for both ecological and economic purposes.

A proteomic approach is thought to be one of the most efficient ways to reveal the molecular biological characteristics of kelp species. Proteomics is a rather novel technical field dealing with the comprehensive analysis of proteins existing in cells or tissues. One of the major tools in proteomics is two-dimensional electrophoresis (2-DE), in which all proteins existing in a cell or a tissue are separated two-dimensionally, and their expression levels are estimated by measuring the spot volumes (integration of spot density over spot area). Proteins in the spots are generally identified by a combination of mass spectrometry and database searching. The 2-DE based proteomics can reveal the molecular biological characteristics of cells or tissues related to various cellular activities such as metabolism, photosynthesis, organization of cytoskeletons, and resistance to environmental stresses. This method can also provide information on the post-translational processing of proteins, such as chemical modifications on amino acid side chains, and limited digestions at peptide bonds, since differentially modified proteins can be separated on the 2-DE gel. Considering that the post-translational processing is often essential to functions of many proteins, this is an important advantage of the 2-DE based proteomics. There are, however, limited numbers of proteomic studies on algae species and no studies involving the investigation of post-translational processing. In this study, in order to reveal molecular biological characteristics of kelp species, proteomic profiles of *Ecklonia kurome* Okamura, one of the Japanese dominant laminarialean kelps, were investigated by 2-DE and a matrix-assisted laser desorption time of

flight/time of flight (MALDI-TOF/TOF) mass spectrometry. Further, we tried to reveal the post-translational processing of some proteins that are thought to be involved in the original biological mechanism of kelp species.

In a previous study (Nagai et al. 2008), as a fundamental tool for proteomic research on kelp species, we developed a novel protein extraction method, named the ethanol/phenol extraction method, in which kelp tissue was directly homogenized in ice-cold ethanol, followed by protein extraction from the resultant precipitates by a phenol/sodium dodecyl sulfate (SDS) method (Wang et al. 2002). This method produced high quality protein powder from the laminae of *E. kurome*. The powder enabled the production of higher-quality one-dimensional electrophoresis or 2-DE gel images and a greater number of bands or spots than other commonly used extraction methods. To the best of our knowledge, this is the first protein extraction method that has been successfully applied to the proteomic analysis of laminarialean kelp.

Subsequently, we performed further investigation to identify *E. kurome* proteins using MALDI-TOF/TOF. At present, there is almost no genomic information for *E. kurome* in the public databases. Therefore, cross-species database searching using peptide mass fingerprinting (PMF) and Mascot MS/MS ion search (MIS) (Perkins et al. 1999) was performed in a previous study, but only 27 out of the 80 analyzed spots were identified. The remaining 53 spots were not identified by these methods, although they showed high quality mass and tandem mass (MS/MS) spectra. The above findings suggested the need for other identification techniques based on sequence similarity searching for the further identification of *E. kurome* proteins. One of the most promising approaches appears to be a mass spectrometry based BLAST (MS BLAST) database searching protocol developed by Shevchenko et al. (2001). MS BLAST is a sequence similarity searching tool optimized for peptides sequence candidates produced by *de novo* interpretation of MS/MS spectra of peptides (*de novo* sequence). It has already been successfully used for the identification of proteins from organisms for which no or limited DNA sequence data are available, such as an extremely halotolerant cyanobacterium *Eubacterium* sp. BAA001 (Pandhal et al. 2008), a green alga *Dunaliella salina* (Dunal) Teodoresco (Waridel et al. 2007), the Holm Oak *Quercus ilex* L. (Jorge et al. 2005), and a fungus *Puccinia triticina* Eriksson (Rampitsch et al. 2006).

In this study, we performed further identification of *E. kurome* proteins using a combination of three database-searching algorithms: Mascot PMF, Mascot MIS, and MS BLAST. The combination of these three algorithms successfully identified many protein spots in 2-DE gels, revealing distinctive characteristics of the *E. kurome* lamina proteome. In particular, vanadium-dependent bromoperoxidases (vBPOs), which is thought to be involved in halogen uptake (Küpper et al. 1998), synthesis of halogenated products (Barre et al. 2010), and detoxification of reactive oxygen species (ROSs) (Küpper et al. 2008, Ritter et al 2010), were found to be highly abundant and separated into more than 20 spots. Furthermore, we partly explained the post-translational processing that caused the heterogeneity of the vBPO spots by more detailed mass analyses and in-gel haloperoxidase activity assay, and discussed the contribution of the processing to the resistance to environmental stresses.

Materials and Methods

Materials

A sporophyte of *E. kurome* collected from the shore in the vicinity of Shirahama (Wakayama Pref., Japan) in October 2007 was washed thoroughly with distilled water and frozen at -80°C. All subsequent steps were carried out at 4°C or on ice, unless otherwise stated. All chemicals used were purchased from Nacalai Tesque (Kyoto, Japan).

2-DE

Proteins were extracted from the lamina of the sporophyte by the ethanol/phenol method (Nagai et al. 2008), and dissolved in 2-D rehydration solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate, 2 mM tri-n-butylphosphine, 0.5% IPG buffer (GE healthcare), and trace of

bromophenol blue. Protein concentrations were determined by the modified Bradford-HCl assay using γ -globulin as a standard (Ramagli and Rodriguez 1985). 2-DE was performed according to a method previously reported (Nagai et al. 2008), using immobilized pH gradient gels (Immobiline DryStrip, pH 4-7, $130 \times 3 \times 0.5$ mm; GE Healthcare, Little Chalfont, UK) for the first dimension, and 10% polyacrylamide gel for the second dimension. Gels were stained with Coomassie brilliant blue (CBB) R-250. Gel images were obtained using Alphaimager (Alpha Innotech, San Leandro, CA, USA) in the transillumination mode. Image analysis of the 2-DE gels was performed using Progenesis PG220 (Nonlinear Dynamics, Newcastle, UK).

MALDI sample preparation

All steps of MALDI-sample preparation, picking up of gel spots, destaining of the gel pieces, and in-gel trypsin digestion, were performed using an Xcise robotic protein processing system (Shimadzu Biotech, Kyoto, Japan), according to a method previously reported (Nagai et al. 2008). The obtained peptides were purified and concentrated using ZipTipTM μ C18 (Millipore, Bedford, MA, USA). Peptides adsorbed in the gels were directly eluted into the MALDI sample plate with 2.5 mg/ml α -Cyano-4-hydroxycinnamic acid (Waters, Milford, MA, USA) in 70% acetonitrile containing 0.1% Trifluoroacetic acid. Mass spectrometric analysis was performed using a 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). MS spectra were measured in the reflector mode in the mass range from 700 to 3500 Da. Data were subjected to external calibration with five standard peptides (Sigma, St. Louis, MO, USA). The MS/MS spectra were measured in collision-induced dissociation mode. Data were subjected to external calibration with fragment peaks of the human adrenocorticotrophic hormone peptide 18-39 (MH^+ 2,465.1989, Sigma).

Strategy for cross-species protein identification

All of the sequential steps of cross-species protein identification used in this study are illustrated in Fig. 1. First, PMF was performed using MASCOT search engine ver 2.1 (Matrix Science, London, UK) against the NCBI nonredundant protein sequence database (www.ncbi.nlm.nih.gov), with a mass tolerance of 0.2 Da. No restrictions were imposed for taxonomy; one possible missed cleavage for trypsin digestion was allowed; and variable modifications such as carbamidomethylation and oxidation of methionine were taken into consideration. Only results with significant MOWSE scores exceeding 79 ($p < 0.05$) were considered as positive identification hits. If only weak peaks were matched, the identified proteins were rejected as probable false-positive hits.

Subsequently, MS/MS spectral data were analyzed using MASCOT MIS, with a precursor mass tolerance of 0.2 Da and MS/MS tolerance of 0.2 Da. Other parameters were the same as for PMF. Positive identification was assigned only if the total ion score exceeded 52 ($p < 0.05$) and at least one of the five most intense peaks were matched.

Finally, homology-based database searching was carried out using MS BLAST against the nonredundant nrdb95 protein database at <http://dove.embl-heidelberg.de/Blast2/msblast.html> as described by Shevchenko et al. (2001). Prediction of amino acid sequences from MS/MS data and generation of queries for MS BLAST were carried out using a DeNovo Explorer ver. 1.22 (Applied Biosystems) with MS/MS tolerance of 0.2 Da. Ten sequences were predicted from each MS/MS spectrum and all candidate sequences generated from one spot were assembled into a single query and submitted to MS BLAST. We considered it as a positive hit when a result of a *de novo* sequence and MS BLAST satisfied the following requirements: (1) prediction of the matched sequences was appropriate, judging from the assignment of b- or y-ion series; (2) scores of high scoring pairs (HSPs) exceeded the threshold value determined by the numbers of HSPs and unique peptides in the query (Shevchenko et al. 2001); and (3) at least one of the five most intense peaks matched.

We categorized the identification results on the basis of the algorithm(s) that enabled the identification: (1) In cases where PMF gave a positive hit, the identification result was classified into the category of PMF; (2) In cases where PMF did not give a positive hit but MIS did, the result was classified as MIS. (3) In cases where a protein candidate identified by PMF or MIS with only minor peaks was also identified

by MS BLAST, this result was classified as a combined search. (4) If only MS BLAST gave a positive hit, the identification result was classified as MS BLAST.

Haloperoxidase activity assay

Detection of haloperoxidase activity on a SDS-polyacrylamide gel electrophoresis (PAGE) gel was performed according to the method of Colin et al with some modifications (Colin et al. 2003). Lamina of *E. kurome* (0.5 g) was homogenized in 2 ml of the 2D-rehydration solution. After the extract was centrifuged at $10,000 \times g$ for 10 min, 10 μ l of the supernatant was subjected to SDS-PAGE using 7.5% polyacrylamide gel. The gel was then soaked with 100 mM potassium phosphate buffer (pH6.0) containing 0.1 mM NaVO_3 , 0.1 mM 3, 3'-dimehoxybenzidine, and 0.45 mM H_2O_2 for 20 min. The assay was started by adding KBr or KI solution (final concentration of 10 mM) and incubated for 30 – 120 min to reveal bromoperoxidase or iodoperoxidase activity, respectively.

Results

2-DE map of lamina of *E. kurome*

Proteins (300 μ g) extracted from lamina of *E. kurome* were separated by 2-DE (pI 4-7, 10% polyacrylamide gel) and detected by CBB staining. A representative 2-DE gel image is shown in Fig. 2b. By image analysis, more than 700 spots were detected in the gel. Among them, 112 abundant and well-defined spots indicated in Fig. 2a were analyzed by MALDI-TOF/TOF, and the resulting mass spectrometric data were used for the cross-species database searching described in the Materials and Methods section. As a result, 93 spots (24 proteins) were successfully identified. Identified proteins with the highest Mascot or MS BLAST scores are summarized in Table 1. In cases of multiple spots that had the same molecular weight and different pI values while showing

similar MS or MS/MS spectra and matched to the same protein, these spots are presented in the same row with the highest Mascot or MS BLAST scores.

Categorization of the analyzed spots according to how they were identified

As shown in Fig. 3a, the 112 analyzed spots were classified according to how they were identified. Among the analyzed spots, 23% (26 spots, 6 proteins) were successfully identified by PMF. The sequence coverage of the identified spots was between 28% and 43%. In all cases, the same proteins were identified by MIS and MS BLAST. MIS also successfully identified 3% of the analyzed spots (3 spots, 3 proteins). The same candidate proteins were also identified by MS BLAST. The combined search and MS BLAST search enables the reliable identification of 48% (54 spots, 11 proteins) and 8% (10 spots 5 proteins) of the analyzed spots, respectively. All of the candidate proteins identified by the PMF and MIS searches with the highest scores belong to macro- or microalgae. Among the 16 proteins identified by the combined and the MS BLAST search, six proteins belong to algal species and the other ten proteins belong to non-algal species, such as fungi, seed plants, protozoa, mollusks, and fish.

Functional annotation of the proteins of *E. kurome*

On the basis of the annotations from Uni-Prot or TrEMBL, 93 identified spots were classified according to their biological functions (Fig. 3b). Since the biological function of vanadium-dependent bromoperoxidase (vBPO) has not been completely revealed yet, we categorized this protein as a haloperoxidase for the sake of convenience. Out of the 112 analyzed spots, 24% are involved in metabolism, 21% belong to the haloperoxidases, and 19% are related to photosynthesis. Others are involved in protein synthesis (6%), protein folding and stabilization (5%), the cytoskeleton (5%), resistance to oxidative stress (2%), and signal transduction (1%). Most of the proteins in the metabolism category are cytoplasmic enzymes involved in carbohydrate

metabolism, such as glucose-6-phosphate isomerase involved in the glycolysis pathway, and 6-phosphogluconate dehydrogenase in the pentose phosphate pathway. Although the 23 spots that belong to the haloperoxidase category exhibit various molecular weights (60-72 kDa) and pI (4.9-5.7) values, they were all identified as homologues of vBPO2 of *Laminaria digitata* (Hudson) J.V.Lamouroux. As shown in Fig. 4a, the 23 spots were divided into 6 groups based on their molecular weight values (a: 72 kDa, b: 68 kDa, c: 60 kDa, d: 73 kDa, e: 70 kDa, f: 63 kDa). The category of photosynthesis consists of proteins localized in the chloroplast and involved in photosynthesis, for example, phosphoribulokinase, which catalyzes the synthesis of D-ribulose 1,5-bisphosphate from D-ribulose 5-phosphate, and ATP synthase subunits a and b, which participate in ATP synthesis in the chloroplast.

Haloperoxidase activity assay

Haloperoxidases extracted from the lamina of *E. kurome* were analyzed by SDS-PAGE (7.5% polyacrylamide gel), followed by an in-gel activity assay (Fig. 4b). At least four major bands (referred to as BPO1-4) were revealed in the presence of either bromide or iodide. One weak band (referred to as IPO) specific for the oxidation of iodide was apparent. The molecular weights of the haloperoxidases in the bands were estimated as follows: BPO1, 68 kDa; BPO2, 62kDa; BPO3, 60 kDa; BPO4, 55kDa; and IPO, 47kDa. According to the estimated molecular weight values, BPO1, 2 and 3 were thought to correspond to BPOb, f, and c detected in the 2-DE gel (Fig. 4a), respectively. The HPO activity of BPO1 was weaker than those of BPO2 and 3, although the level of expression of BPOb was higher than those of BPOf and c. No HPO activity was detected around 70 kDa, where BPOa, d and e were present, while HPOs corresponding to BPO4 and IPO were not detected in the 2-DE gel.

Comparison of vBPO spots by MALDI-TOF/TOF

In order to investigate difference among the six vBPO groups shown in Fig. 4a, mass spectra of their tryptic digests were compared. Figure 5a shows representative mass spectra of the six groups. Spots belonging to the same group gave almost the same mass spectra (data not shown). BPOa, b, and c showed similar mass spectra, in which many peaks such as m/z 1651, 1991, and 1529 were commonly observed. However, they showed the following differences: (1) a peak at m/z 1325 was observed in BPOa and b, but not in BPOc; (2) a peak at m/z 2187 was observed only in BPOa; and (3) a peak at m/z 1945 was observed in BPOb and c, but not in BPOa. In the same way, BPOd, e, and f showed similar mass spectra, but also showed the following differences: (1) a peak at m/z 1370 was observed in BPOd and e, but not in BPOf, (2) a peak at m/z 2213 was observed only in BPOd; and (3) a peak at m/z 2112 was observed in BPOe and f, but not in BPOd. Since mass spectra of BPOa-c and BPOd-f were markedly different, they are suggested to be derived from two different vBPO isoforms.

Amino acid sequences of the differential peaks were predicted by *de novo* interpretation (Fig. 5c). As an example, an MS/MS spectrum of the peak at m/z 2187 observed only in BPOa is shown in Fig. 5b with the predicted sequence. As shown in Fig. 5b, y-series and b-series ions were extensively detected and they complemented each other in all cases of the prediction. Predicted sequences of the four differential peaks, m/z 2187 in vBPOa, m/z 1945 in vBPO b and c, m/z 2213 in BPOd, and m/z 2112 in vBPO e and f, showed higher similarity to the N-terminal region of *L. digitata* vBPO2. The sequences of m/z 2187 and 1945 were the same, except that two N-terminal residues (KN) in m/z 2187 were absent in m/z 1945. In the same way, the sequences of m/z 2213 and 2112 were the same, except that an N-terminal residue (T) was absent in m/z 2112. These results strongly indicated that vBPOb and e were produced by limited cleavage at the N-terminal region of vBPOa and d, respectively. The cleavage site in vBPOa was predicted to be a peptide bond between asparagine 2 and glutamate 3 in the sequence of KNEPPFEKTQPLLSGVCGR, while that in vBPOd was predicted to be a peptide bond between threonine 1 and valine 2 in the sequence of TVDLNEKVVQPLLSGVCGR.

In addition, the predicted sequences of m/z 1324 in vBPOa and b, and m/z 1369 in vBPOd and e were homologous to the sequence in the C-terminal region of *L. digitata* vBPO2. This also indicated that vBPOc

and f were produced by digestion at the C-terminal region of vBPOb and e, respectively, although the precise digestion sites were unclear.

Discussion

Homology-based protein identification of *E. kurome*

In this study, proteomic analysis of an unsequenced macroalga, *E. kurome* was conducted by 2-DE, MALDI-TOF/TOF, and cross-species protein identification using a combination of three database searching algorithms, Mascot PMF, MIS, and MS BLAST. Mascot PMF and MIS are one of the most widely used and effective techniques for protein identification. However, they have limitation in terms of cross-species protein identification, since they rely on stringent matching of peptide masses to the corresponding peptide masses obtained by *in silico* processing of protein sequences from database entries. Although these algorithms can detect peptides with sequences identical to those in known databases with high sensitivity and specificity, they cannot detect non-identical but highly homologous peptides. Sequence similarity searching tools optimized for peptide sequences produced by *de novo* interpretation of MS/MS spectra, such as MS BLAST, could overcome this issue and enhance the sensitivity of cross-species identification. In our previous study, only 33.8% of the analyzed spots (27 out of 80 spots) of *E. kurome* proteome were identified using MALDI-TOF/TOF and Mascot search. Using the same method, Contreras et al. (2008) analyzed 46 protein spots from *Scytosiphon gracilis* and 150 spots from *Ectocarpus siliculosus*, but identified only 10 (21.7%) and 14 (9.3%) spots, respectively. More recently, Kim et al. (2011) analyzed 39 protein spots from *Saccharina japonica* and identified 15 of them (39.4%). In these previous reports, many spots could not be identified by conventional database searching. In contrast, in the present study, a combination of three algorithms, Mascot PMF, MIS, and MS BLAST, successfully identified as many as 83% of the analyzed spots (93 out of 112 spots) of the *E. kurome* lamina proteome. Among them, Mascot PMF and MIS identified only 26% of the analyzed spots, and the

taxonomic origin of the matching sequences was restricted to micro- and macro-algae. However, the introduction of MS BLAST expanded the taxonomic origin of the matching sequences to a wide variety of species, such as fungi, seed plants, protozoa, mollusks, and fish, which markedly increase the identification rate by 57%. These results demonstrate the efficiency of the approach combining Mascot and MS BLAST for the proteomic analysis of an unsequenced macro-algal species.

In many cases, the same protein was identified in multiple spots with the same molecular weight and different *pI* values. For example, glyceraldehyde-3-phosphate dehydrogenase (G3PD), with a weak alkaline theoretical *pI* value, was identified in eight different spots located in a more acidic *pI* region. These *pI* shifts probably reflect differences in post translational modifications. In fact, recent mass spectrometric analyses have identified many modifications such as phosphorylation and acetylation in human G3PD (Seo et al. 2008).

There remained 17% of spots that were not identified, even though most of them showed a high-quality mass or MS/MS spectra. This was probably because these proteins are unique to laminarialean kelp, and no similar sequences have yet been registered in public protein sequence databases.

Proteomic profile of lamina of *E. kurome* and investigation of the heterogeneity of haloperoxidases

Our results of cross-species protein identification of the *E. kurome* proteins revealed distinctive characteristics of the *E. kurome* lamina proteome. As shown in Fig. 3b, a significant proportion of the proteins belong to metabolic enzymes (24%), haloperoxidases (21%), and photosynthetic proteins (19%). An abundance of metabolic and photosynthetic proteins is a common finding in 2-DE reference maps of land plants, as previously reported (Jorge et al. 2005; Maldonado et al. 2008; Albertin et al. 2009; Katam et al. 2010). However, the remarkable abundance and heterogeneity of haloperoxidases were not reported in those 2-DE maps. In 2000, a set of 500 expressed sequence tags (ESTs) were generated from sporophytes and gametophytes of *Laminaria digitata*, and 152 different proteins were identified (Crépineau F et al, 2000). The distribution of the cellular roles of the identified proteins differed markedly from our data. In this earlier data, the majority of identified ESTs from

sporophytes of *L. digitata* were involved in gene or protein expression (38.7%), and the second most abundant class of ESTs encoded enzymes involved in cellular metabolism (3.9%). Only 1% of the identified ESTs belonged to the category of cell/organism defense which included vBPO. The discrepancy between their results of EST analysis and our proteomic data is probably derived from the fact that the level of a protein is not always proportional to the expression level of its corresponding mRNA, since this relationship can be affected by many factors including transcriptional regulation, post-translational modification or degradation of the protein, and endo- or exocytosis.

Haloperoxidases, a family of enzymes that catalyze the oxidation of halides by hydrogen peroxide, have been discovered in many marine organisms. In particular, vanadium-dependent haloperoxidases (vHPOs) appear to be the most prevalent (Butler et al. 2001). vHPOs are named according to the most electronegative halide that they can oxidize. Thus, chloroperoxidase can catalyze the oxidation of chloride as well as of bromide and iodide, bromoperoxidase (vBPO) can catalyze the oxidation of bromide and iodide, and iodoperoxidase (vIPO) is specific for the oxidation of iodide. To date, only vBPOs and vIPOs have been detected in algal species. The vBPOs and vIPOs catalyze the oxidation of bromide and iodide to generate oxidized halogens, which are thought to be related to iodine uptake in laminarialean algae (Küpper et al. 1998). In the kelp *L. digitata*, the accumulated iodine were found to be mainly stored in the apoplastic region as iodide (Verhaeghe et al. 2008), which showed the powerful reducing activity to a wide variety of ROSs (Küpper et al. 2008). More recently, Ritter et al found that copper stress markedly increased the expression of vBPO in the copper tolerant strains of *Ectocarpus siliculosus* (Ritter et al. 2010). Considering that the Cu^{2+} can catalyze the synthesis of the highly reactive hydroxyl radical in presence of H_2O_2 via the Fenton reaction, their result also indicated the importance of vBPO in the detoxifications of ROSs.

In the present study, we identified 23 spots as homologous to vBPO of *L. digitata*, a close relative of *E. kurome*. In fact, in-gel HPO activity assay (Fig. 4b) indicated that the three vBPO groups (vBPOb, d, and f shown in Fig. 4a) had both BPO and IPO activities. Similarity of mass spectra of the tryptic digests suggested that they are derived from two different vBPO isoforms (vBPOa-c, and vBPOd-f). It has been reported that some

brown algae have different vBPOs (Krenn et al. 1989; Colin et al. 2003). The two vBPOs of *E. kurome* might be involved in different physiological functions. In gel HPO activity assay also revealed that three out of the six vBPO groups (vBPOa, d, and e) had neither BPO nor IPO activities. MS/MS analysis revealed that the differences between the inactive vBPOs and smaller active vBPOs were related to the presence or absence of one or two residues in the N-terminal and C-terminal sequences (Fig. 5). From these results, the activation process of *E. kurome* vBPOs was suggested to occur as follows: (1) vBPOa and d are the inactive forms, (2) digestion at the N-terminal region converts the immature vBPOs into weakly active or inactive intermediates (vBPOb and e), and (3) subsequent digestion at the C-terminal region converts the intermediates into the active mature forms (vBPOc and f). Many enzymes have already been shown to be activated through limited proteolysis at N-terminal or C-terminal regions. For example, it is well known that chymotrypsinogen is converted into a mature form called α -chymotrypsin by limited cleavage at the N-terminal region and, subsequent digestion at the C-terminal region (Berg et al. 2002).

It should be noted that large amounts of pro-vBPOs are expressed in the *E. kurome* lamina. It is probably the case that *E. kurome* usually stores pro-vBPOs in its lamina, so that it can rapidly respond to environmental stresses such as ROSs. However, in our HPO activity assay, protein extraction and the subsequent SDS-PAGE were performed under denaturing conditions. Considering that the assay can detect only HPO molecules that successfully refold in the gel, there is a possibility that the result of Fig. 4b does not reflect the actual activities of the HPOs in vivo. The relationship between the N- or C-terminal digestion and the activities of the vBPOs should be investigated in more detail in future studies.

In conclusion, the present study demonstrated the efficiency of homology-based cross-species protein identification for the proteomic analysis of unsequenced laminarialean kelps. This approach revealed the distinctive biological characteristics of *E. kurome* lamina, and shed some light on the mechanism of resistance to environmental stresses. The methods used in this study should facilitate the molecular biological investigation of kelp and other macro-algal species.

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Figure Captions

Fig. 1 Scheme for the homology-based cross-species protein identification used in this study.

Fig. 2 2-DE map of *Ecklonia kurome* lamina extract with (a) and without (b) labels of analyzed spots. Proteins (300 µg) extracted from the lamina of *E. kurome* were separated by 2-DE (pI 4-7, 10% polyacrylamide gel) and detected by CBB. Protein spots significantly identified are numbered in accordance with Table 1. Protein spots that were not identified are circled by dotted lines.

Fig. 3 Categorization of the analyzed spots according to the identification methods (a) and the biological functions of the identified proteins (b). Biological functions were estimated from annotations in the Uni-Prot or TrRMBL databases.

Fig. 4 (a) A focused image of vanadium-dependent bromoperoxidase (vBPO) spots in the 2-DE gel. The vBPO spots were classified into 6 groups according to their molecular weights: vBPOa, 72 kDa; vBPOb, 68 kDa; vBPOc, 60 kDa; vBPOd, 73 kDa, vBPOe, 70 kDa; and vBPOf, 63 kDa. The vBPOa, b,c,d,e and f correspond to spots No 6-10, 11-14, 18-22, 27-30, 31-34, and 35 in the Fig 2a, respectively. (b) In gel haloperoxidase activity assay of the protein extracts from lamina of *Ecklonia kurome*. Lane 1 shows bromoperoxidase activity bands detected by 30 min incubation. Lanes 2 and 3 show iodoperoxidase activity bands detected by 30 min and 120 min incubation, respectively. Bromoperoxidase activity bands are referred to as BPO 1-4, and an iodoperoxidase activity band is indicated as IPO. M: Molecular mass markers.

Fig. 5 *De novo* interpretation from MS/MS spectra of the distinctive peptides of the six vBPO groups. (a)

Representative mass spectra of tryptic digests of proteins in the six vBPO groups shown in Fig. 4A. Numbers in bold font indicated the m/z values of the peaks that were matched to the vBPO2 of *Laminaria digitata*. Numbers with gray squares correspond to the m/z values of the peaks distinctive for the 6 vBPO groups. *: trypsin autolysis peptides. (b) A MS/MS spectrum of a peak at m/z 2187.0 in vBPOa with a predicted amino acid sequence. (c) Predicted amino acid sequences of the 6 distinctive peaks, along with the corresponding sequences in vBPO2 of *L. digitata*.

Table 1. List of *E. kurome* proteins identified by MALDI-TOF/TOF and homology-based cross-species database searching.

Spot No.	Protein Name	Species	Accession ID (NCBI)	Accession ID (EMBL)	MW (theoretical / obs.)	pI (theoretical / obs.)	Function	Type of Identification	PMF score (NPM*, SC**)	MIS score (NPM*)	BLAST score (NHSPs***)
1	Heat shock protein 90 (Fragment)	<i>Mallomonas rasilis</i> (microalga)	gi 59894150	AAX10943	65508 / 96 k	5.26 / 4.7	Protein folding and stabilization	MIS	-	245 (3)	227 (4)
2-4	BiP/GRP78	<i>Aplysia californica</i> (protozoan)	gi 5570	CAA78759	73653 / 78 k	4.80 / 4.7	Protein folding and stabilization	Combined	-	111 (2)	751 (12)
5	Heat shock protein 70	<i>Stylonychia lemnae</i> (protozoan)	gi 7141314	-	71165 / 78 k	5.5 / 4.9	Protein folding and stabilization	Combined	-	99 (2)	-
	Hsp70 protein (Fragment)	<i>Cetorhinus maximus</i> (fish)	-	AAM53203	50686 / 78 k	6.0 / 4.9	Protein folding and stabilization		-	-	168 (39)
6-10	Vanadium-dependent bromoperoxidase 2	<i>Laminaria digitata</i> (macroalga)	gi 32329414	CAD37192	75078 / 72 k	5.56 / 5.1-5.3	Haloperoxidase	Combined	-	216 (3)	564 (10)
11-14	Vanadium-dependent bromoperoxidase 2	<i>Laminaria digitata</i> (macroalga)	gi 32329414	CAD37192	75078 / 68 k	5.56 / 5.3-5.5	Haloperoxidase	Combined	-	216 (4)	487 (9)
15-17	Glucose-6-phosphate isomerase	<i>Phytophthora infestans</i> (fungus)	gi 37960095	AAP51080	60726 / 64 k	6.04 / 5.7-5.8	Metabolism	Combined	-	63 (2)	92 (1)
	Phosphoglucose isomerase	<i>Dioscorea tokoro</i> (Seed plant)	-	BAA23176	62748 / 64 k	6.29 / 5.7-5.8	Metabolism		-	-	222 (4)
18-22	Vanadium-dependent bromoperoxidase 2	<i>Laminaria digitata</i> (macroalga)	gi 32329414	CAD37192	75078 / 60 k	5.56 / 5.4-5.7	Haloperoxidase	Combined	-	178 (4)	586 (12)
23-26	Vacuolar ATP synthase subunit B	<i>Encephalitozoon cuniculi</i> (protozoan)	gi 19074713	CAD25823	53074 / 59 k	5.12 / 5.2-5.4	Metabolism	Combined	-	87 (1)	137 (2)
	Vacuolar ATPase subunit B	<i>Blastocystis hominis</i> (protozoan)	-	BAA97567	54266 / 59 k	5.14 / 5.2-5.4	Metabolism		-	-	213 (3)
27-30	Vanadium-dependent bromoperoxidase 2	<i>Laminaria digitata</i> (macroalga)	gi 32329414	CAD37192	75078 / 73 k	5.56 / 4.9-5.0	Haloperoxidase	Combined	-	30 (1)	371 (5)
31-34	Vanadium-dependent bromoperoxidase 2	<i>Laminaria digitata</i> (macroalga)	gi 32329414	CAD37192	75078 / 70 k	5.56 / 4.9-5.0	Haloperoxidase	Combined	-	89 (2)	264 (5)
35	Vanadium-dependent bromoperoxidase 2	<i>Laminaria digitata</i> (macroalga)	gi 32329414	CAD37192	75078 / 64 k	5.56 / 4.9	Haloperoxidase	Combined	-	59 (1)	203 (3)
36	Protein disulfide isomerase	<i>Zea mays</i> (Seed plant)	-	AAX09966	47149 / 58 k	5.45 / 4.8	Protein folding and stabilization	BLAST	-	-	144 (3)
37-41	AtpA protein	<i>Pisum sativum</i> (Seed plant)	gi 12143	-	54592 / 52 k	5.75 / 4.7-4.9	Photosynthesis	Combined	-	134 (1)	
	ATP synthase alpha chain	<i>Chlamydomonas reinhardtii</i> (microalga)	-	CAA42840	54621 / 52 k	5.44 / 4.7-4.9	Photosynthesis		-	-	337 (6)

* NPM: number of peptides matched; ** SC, sequence coverage; *** NHPS, number of high-scoring pairs.

Table 1. Continued

Spot No.	Protein Name	Species	Accession ID (NCBI)	Accession ID (EMBL)	MW (theoretical / obs.)	pI (theoretical / obs.)	Function	Type of Identification	PMF score (NPM*, SC**)	MIS score (NPM*)	BLAST score (NHSPs***)
42-46	Beta subunit of ATPase	<i>Pylaiella littoralis</i> (microalga)	gi 14181	CAA42899	51993 / 50 k	5.03 / 4.7-4.9	Photosynthesis	PMF	84 (17, 37%)	372 (5)	319 (5)
	ATP synthase beta chain	<i>Euglena gracilis</i> (microalga)	-	CAA50128	52323 / 50 k	5.13 / 4.7-4.9	Photosynthesis		-	-	348 (6)
47-49	Phosphoglycerate kinase (Fragment)	<i>Laminaria digitata</i> (macroalga)	gi 6453561	CAB61334	37009 / 49 k	4.87 / 4.8-4.9	Metabolism	PMF	79 (9, 34%)	184 (2)	176 (3)
	Phosphoglycerate kinase	<i>Isochrysis galbana</i> (microalga)	-	AAW79325	46307 / 49 k	5.04 / 4.8-4.9	Metabolism				229 (3)
50-56	Elongation factor Tu	<i>Undaria pinnatifida</i> (macroalga)	gi 4001799	AAC94989	40078 / 48 k	4.70 / 4.8-5.0	Protein synthesis	PMF	99 (11, 35%)	299 (3)	211 (3)
	Elongation factor Tu	<i>Chara connivens</i> (macroalga)	-	AAA87685	45321 / 48 k	5.78 / 4.8-5.0	Protein synthesis		-	-	229 (4)
57-61	Phosphoribulokinase	<i>Vaucheria litorea</i> (microalga)	gi 13398515	AAK21910	44777 / 46 k	5.12 / 4.8-5.0	Photosynthesis	Combined	-	87 (1)	207 (3)
62-65	6-phosphogluconate dehydrogenase	<i>Laminaria digitata</i> (macroalga)	gi 6453557	-	57335 / 52 k	6.19 / 5.4-5.6	Metabolism	PMF	82 (16, 28%)	275 (3)	-
	6-phosphogluconate dehydrogenase	<i>Listeria monocytogene</i> (bacteria)	-	CAC99454	52415 / 52 k	5.11 / 5.4-5.6	Metabolism		-	-	182 (3)
66-71	Actin	<i>Laminaria japonica</i> (macroalga)	gi 77745053	-	41764 / 46 k	5.30 / 5.2-5.5	Cytoskeleton	PMF	151 (16, 41%)	372 (4)	-
	Beta-actin	<i>Fucus vesiculosus</i> (macroalga)	gi 1419540	CAA67388	41863 / 46 k	5.39 / 5.2-5.5	Cytoskeleton		138 (15, 43%)	372 (4)	317 (4)
72-74	Fructose-1,6-bisphosphate aldolase class I	<i>Prochlorococcus marinus</i> (microalga)	gi 33237833	AAP99900	38505 / 42 k	6.86 / 5.0-5.1	Metabolism	Combined	-	70 (1)	238 (4)
75-77	Glyceraldehyde-3-phosphate dehydrogenase precursor	<i>Guillardia theta</i> (microalga)	-	AAC49702	41426 / 39 k	8.04 / 6.0-6.2	Metabolism	BLAST	-	-	253 (5)
78-81	Glyceraldehyde-3-phosphate dehydrogenase	<i>Trypanoplasma borreli</i> (protozoan)	gi 520888	CAA52631	39329 / 38 k	9.07 / 5.7-6.1	Metabolism	Combined	-	72 (1)	241 (4)
	Glyceraldehyde-3-phosphate dehydrogenase	<i>Plasmodium berghei</i> (protozoan)	-	AAP33840	36808 / 38 k	7.17 / 5.7-6.1	Metabolism				368 (6)
82	14-3-3	<i>Fucus vesiculosus</i> (macroalga)	gi 1419538	CAA67389	28152 / 30 k	4.50 / 4.3	Signal transduction	PMF	175 (39%)	423 (5)	268 (5)
83	Oxygen-evolving enhancer protein 1 precursor	<i>Phaeodactylum tricornutum</i> (microalga)	-	AAO43192	32630 / 32 k	5.16 / 5.2	Photosynthesis	BLAST	-	-	153 (3)

* NPM: number of peptides matched; ** SC, sequence coverage; *** NHPS, number of high-scoring pairs.

Table 1. Continued

Spot No.	Protein Name	Species	Accession ID (NCBI)	Accession ID (EMBL)	MW (theoretical / obs.)	pI (theoretical / obs.)	Function	Type of Identification	PMF score (NPM*, SC**)	MIS score (NPM*)	BLAST score (NHSPs***)
84-86	Oxygen-evolving enhancer 1	<i>Heterosigma akashiwo</i> (microalga)	-	AAN11311	31943 / 30 k	5.55 / 5.1-5.4	Photosynthesis	BLAST	-	-	365 (7)
87	CfxQ	<i>Odontella sinensis</i> (microalga)	gi1185223	CAA91706	32844 / 31 k	6.17 / 5.8	Photosynthesis	MIS	-	195 (2)	248 (4)
88	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	<i>Azoarcus sp. EbN1</i> (bacteria)	gi56312032	CAI06677	28028 / 29 k	6.24 / 6.2	Metabolism	Combined	-	51 (1)	130 (2)
	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	<i>Erwinia carotovora</i> (bacteria)	-	CAG74292	28423 / 29 k	5.53 / 6.2	Metabolism		-	-	148 (3)
89-90	Short-chain dehydrogenase/reductase	<i>Burkholderia sp. 383</i> (bacteria)	gi77964830	-	25074 / 27 k	6.84 / 5.8-6.1	Metabolism	Combined	-	111 (1)	-
	Putative short-chain dehydrogenase/	<i>Acinetobacter sp. ADP1</i> (bacteria)	-	CAG68662	57265 / 27 k	6.08 / 5.8-6.1	Metabolism		-	-	117 (2)
91-92	Superoxide dismutase, Fe dependent	<i>Idiomarina loihiensis</i> L2TR (bacteria)	-	AAV82637	21635 / 22 k	4.89 / 5.3-5.4	Resistance to oxidative stress	BLAST	-	-	271 (3)
93	Fucoxanthin-chlorophyll a-c binding protein A	<i>Macrocystis pyrifera</i> (macroalga)	gi2498364	AAC49018	22868 / 16 k	5.61 / 4.6	Photosynthesis	MIS	-	156 (2)	125 (2)

* NPM: number of peptides matched; ** SC, sequence coverage; *** NHPS, number of high-scoring pairs.

Figure 1

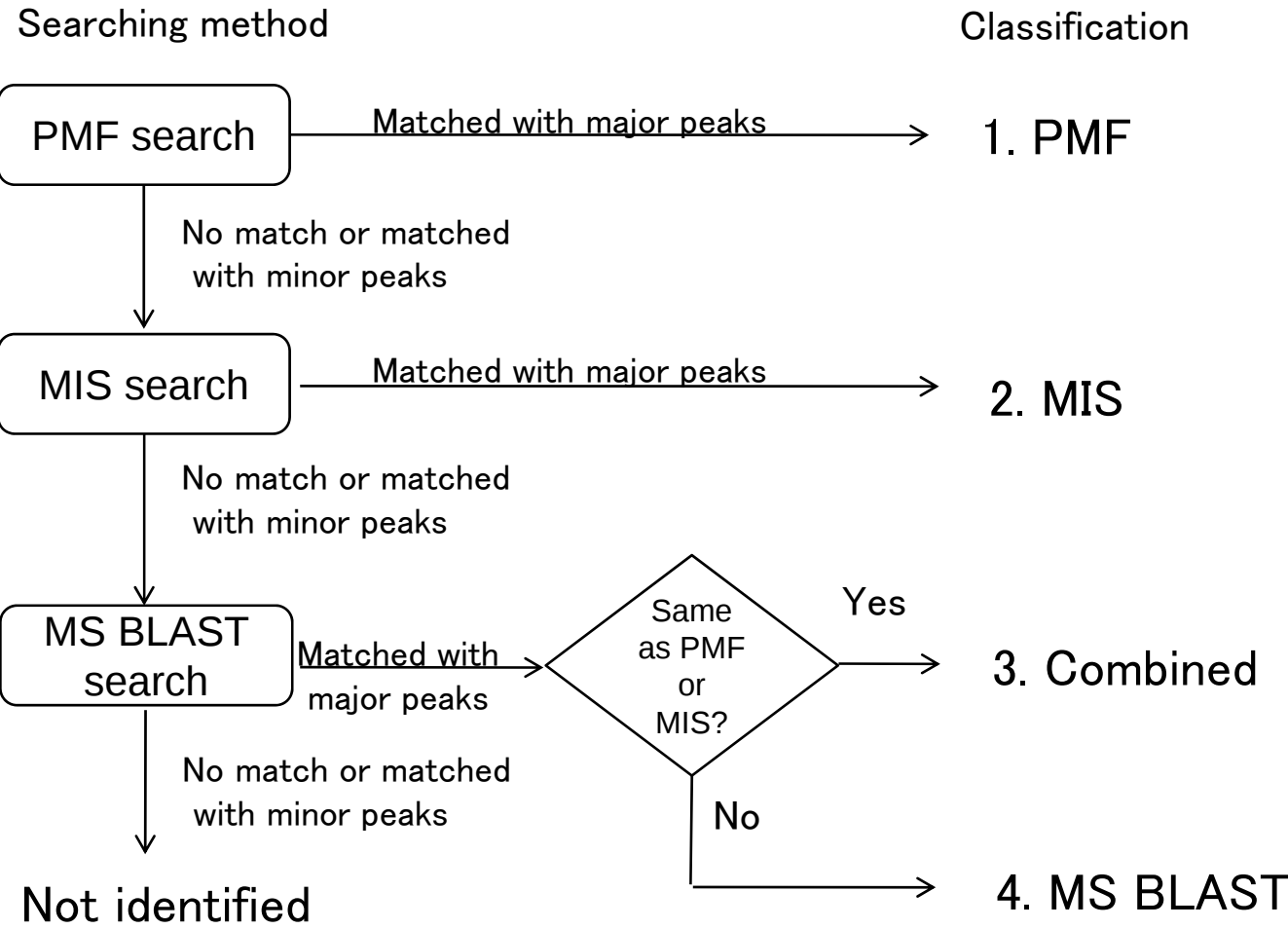


Figure 2

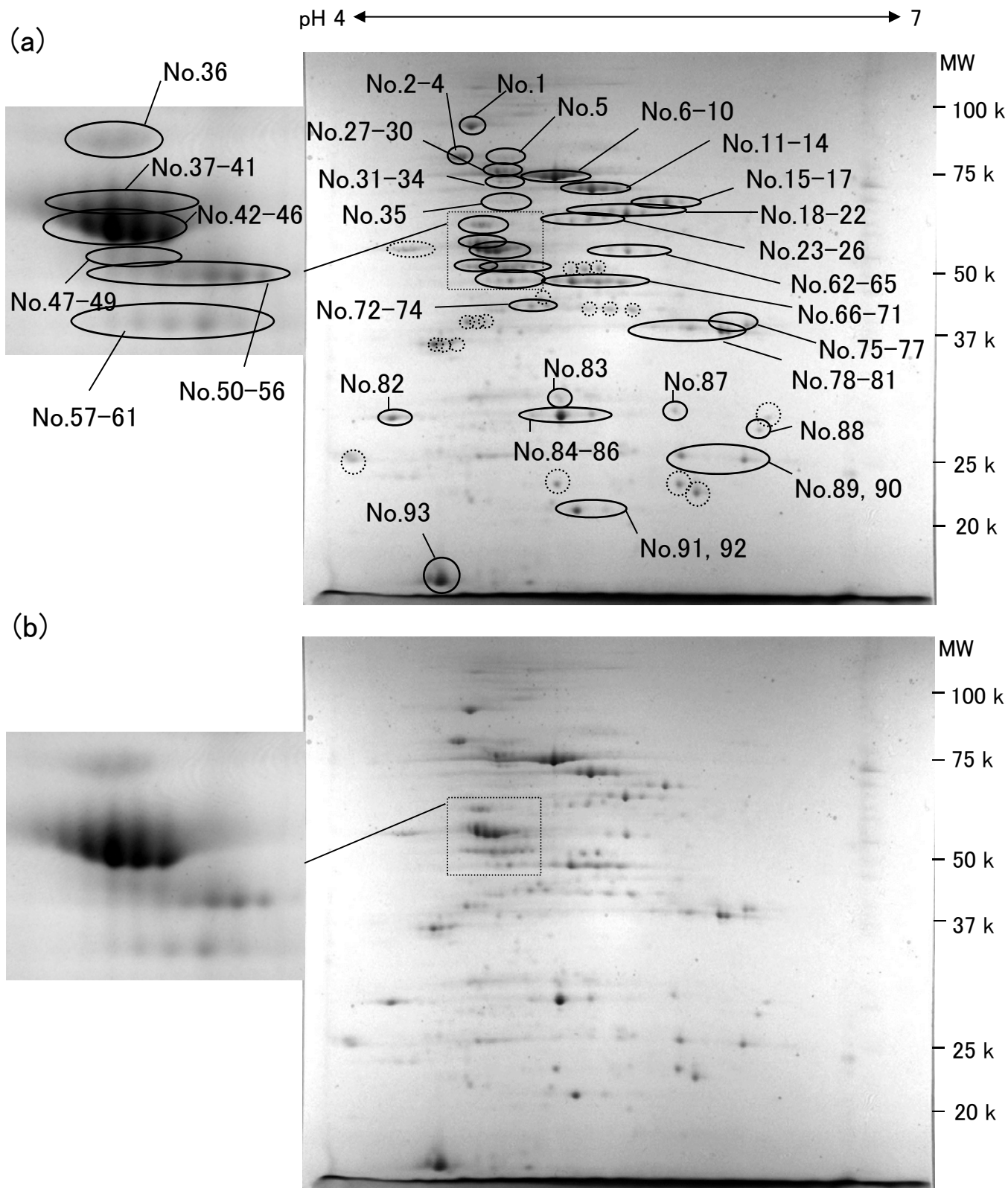


Figure 3

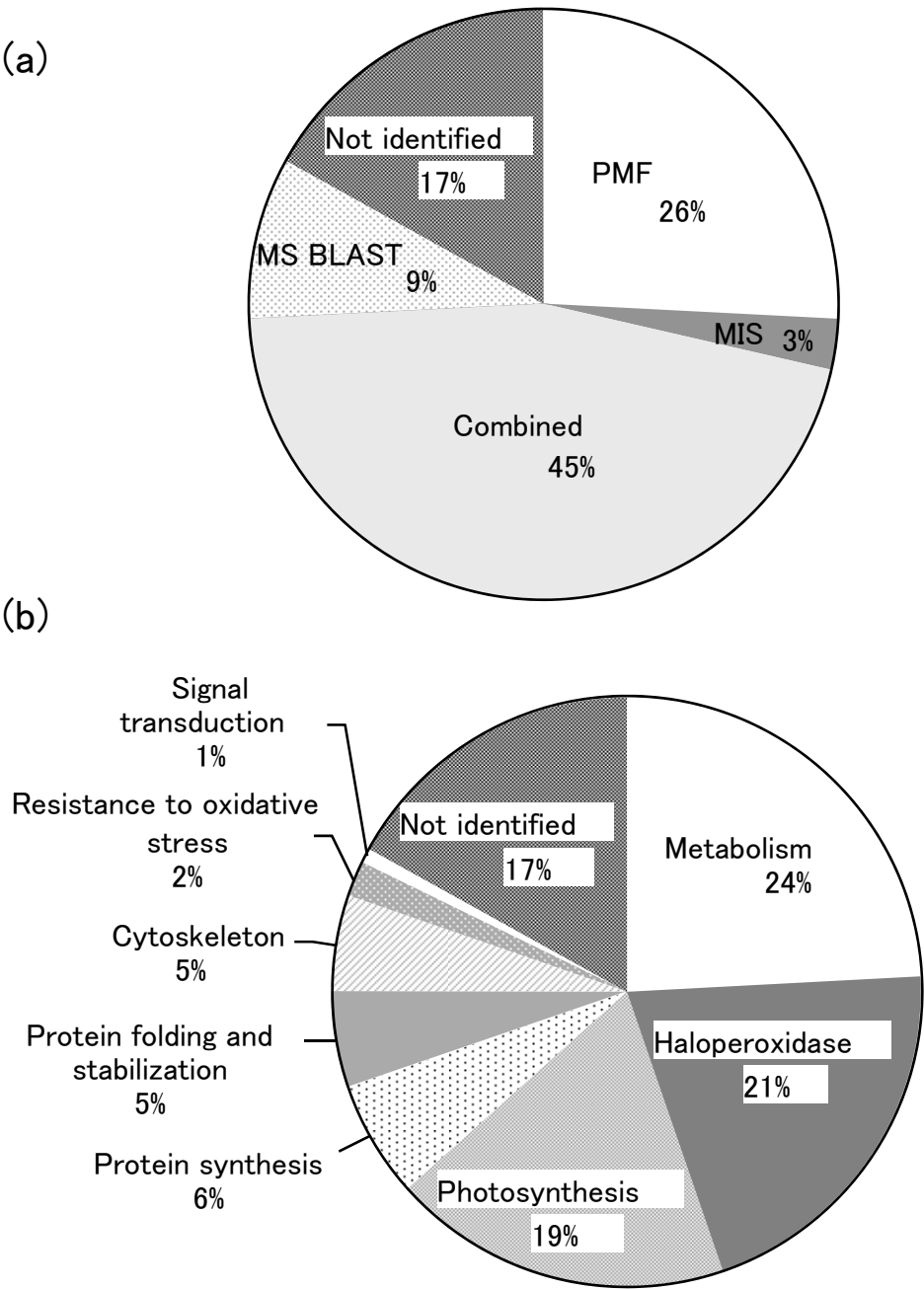
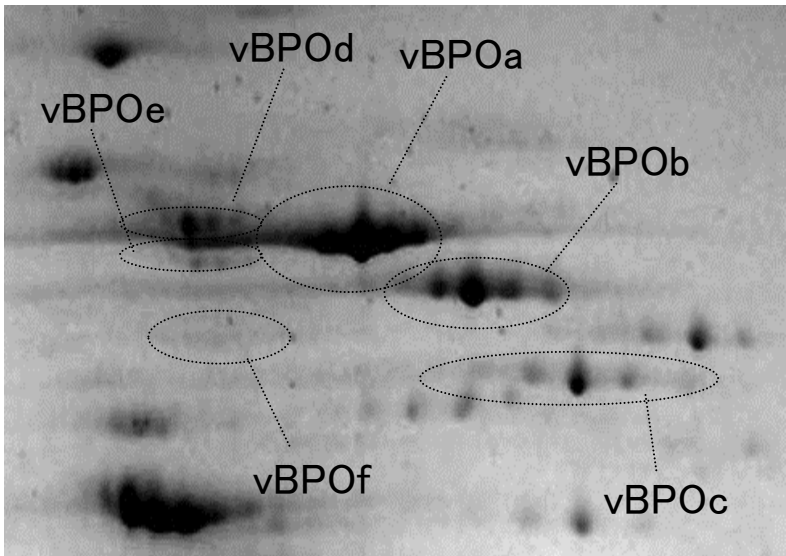


Figure 4

(a)



(b)

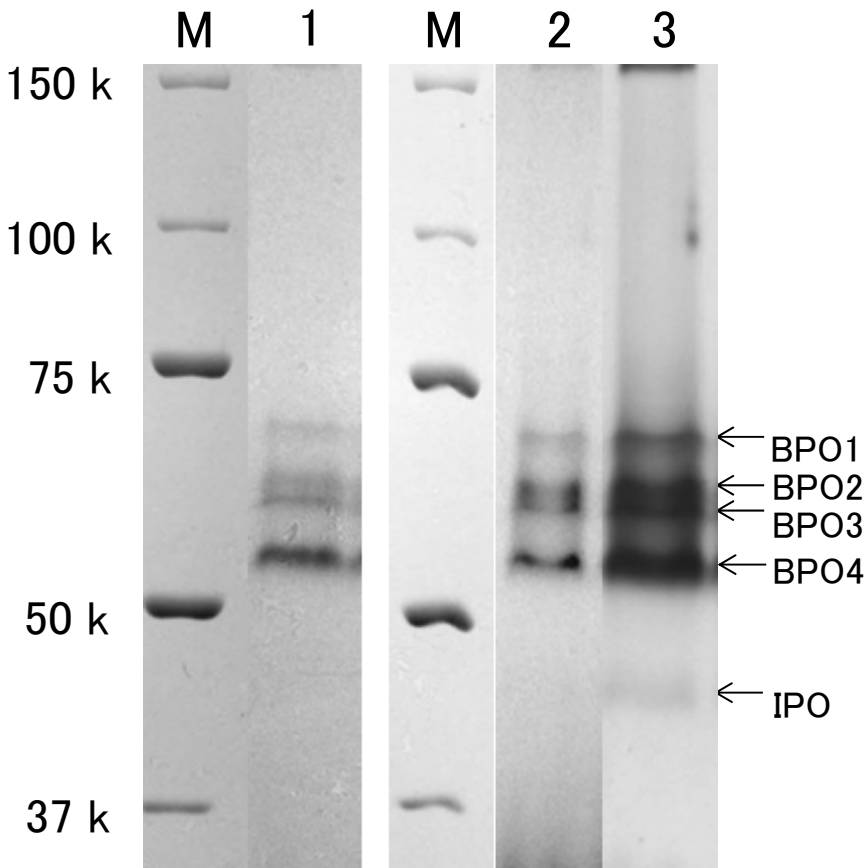
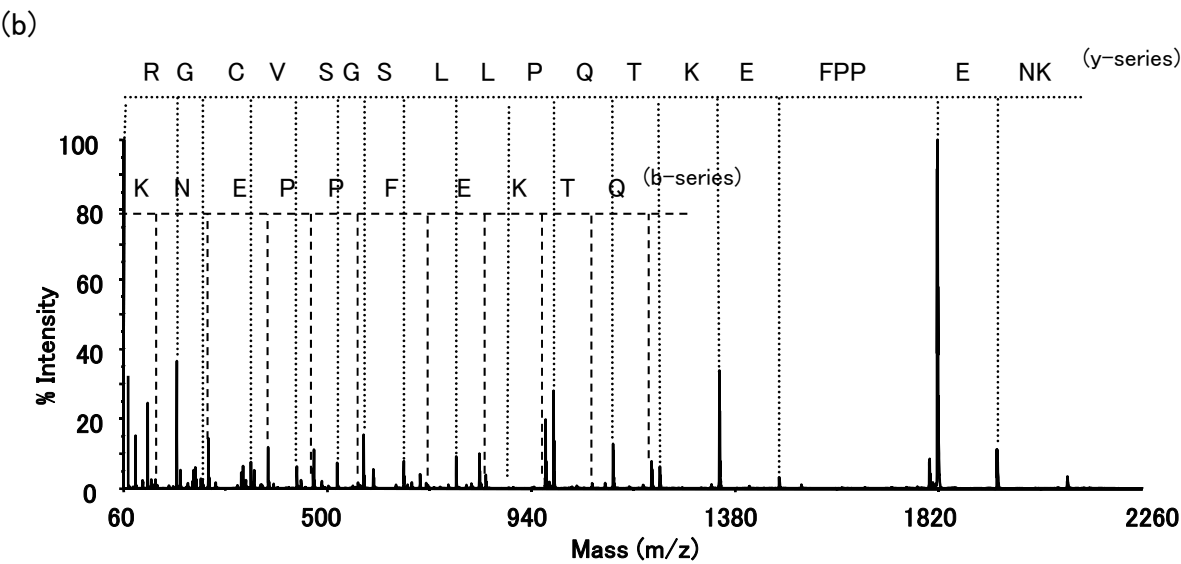
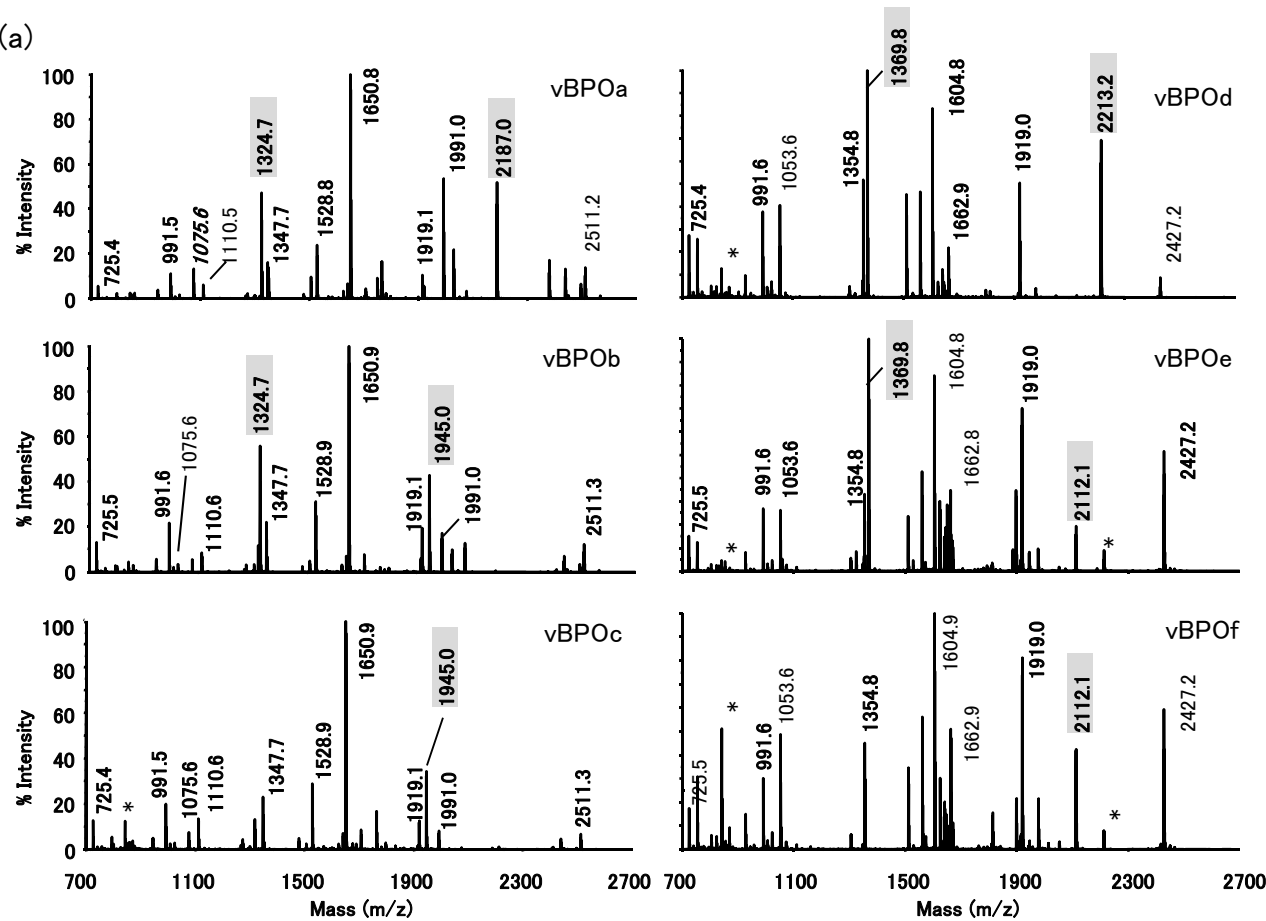


Figure 5



(c)

Residues 58–64 of <i>L. digitata</i> vBPO2	E E P P E T Q P L L S G N V C R
m/z 2187 from BPOa	K N E P P F E K T Q P L L S G S V C G R
m/z 1945 from BPOb and c	E P P F E K T Q P L L S G S V C G R
m/z 2213 from BP Od	T V D L N E V K V V Q P L L S G S V C G R
m/z 2112 from BPOe and f	V D L N E V K V V Q P L L S G S V C G R
Residues 629–636 of <i>L. digitata</i> vBPO2	L L E L Y G R N L Y K
m/z 1324 from BPOa and b	L E L Y G L A N L Y R
m/z 1369 from BP Od and e	K L E L F S S N L Y R