Characterization of recombinant protein ferritin from *Pyropia yezoensis* (rPyFer) and its biological activities

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Abstract Ferritins are iron-binding proteins that are basically participated in iron storage, detoxification, and immune response. In the present study, ferritin gene from the marine red algae *Pyropia yezoensis* was cloned into a pET21d expression vector. High-efficiency transformation was performed in *Escherichia coli* BL21, the recombinant protein was expressed by induction with 0.1 mM isopropyl- β -D-thiogalactoside and purified via ammonium sulfate precipitation, anion exchange and size exclusion chromatography. The purified recombinant ferritin from *P. yezoensis* (rPyFer) was characterized and analyzed for its antimicrobial activity against both Gram-negative and Gram-positive bacterial cultures and exhibited significant antibacterial activity against Gram-positive cultures. The recombinant protein was also analyzed for its iron-uptake and radical-scavenging activities; rPyFer exhibited significant iron-uptake activity at low concentrations, and its radical-scavenging activity increased in a dose-dependent manner. This research will contribute to the development of new therapeutic proteins from marine algae.

Keywords Recombinant protein · *Pyropia yezoensis* · Ferritin · Equine spleen ferritin · Marine algae · Antioxidant activity · Minimum inhibitory concentration

Introduction

Ferritin is an important iron-binding protein which is found in all species. Ferritin is made up of a hollow spherical oligomer consisting of 24 subunits, and iron can be stored within this structure as stable and nontoxic state to fulfil iron requirements (Arosio et al., 2009). In vertebrates, ferritin composed of two homologous subunits, light (L)-ferritin and heavy (H)-ferritin, they have different specialized roles. H-ferritin exhibits ferroxidase activity and participates in rapid iron uptake and detoxification, whereas L-ferritin is involved in long-term iron storage (Ford et al., 1984).

Algae was confirmed to be an excellent source of iron for supplementation. Several types of marine macroalgae are commonly consumed in countries such as Korea, Japan, and China. Among these macroalgae, the red alga *P. yezoensis*, also called nori, is the most important species and contains high levels of iron, zinc, and manganese, as well as folic acid and vitamins A, B12 and C (Takatoshi and Miho, 1993). Although the iron content of *P. yezoensis* has been reported previously (Shaw and Liu, 2000), various brown (e.g., *Sargassum* sp.), green (e.g., *Ulva* sp.), and red (e.g., *Porphyra* sp. and *Gracilariopsis* sp.) marine macroalgae have been reported to have a high content of iron compared to other plant-derived foods, and the iron acquired from these macroalgae is well-absorbed by humans (Garcia-Casal et al., 2007; Goñi et al., 2000; Masuda et al., 2015) examined the various forms of iron in *P. yezoensis* and demonstrated that ferritin acts in the red

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macroalgae as an iron-storage device. Most research on iron storage mechanism of algae has been conducted on prokaryotic and unicellular eukaryotic microalgae. For example, ferritin plays a key roles in cyanobacteria and bloom-forming marine pennate diatoms in iron storage and proliferation (Marchetti and Cassar, 2009). *Chlamydomonas*, a genus of unicellular green algae in the class Chlorophyceae, have multiple genes encoding plant-type ferritins whose expressions are differently regulated under iron deficiency and sufficiency (Long et al., 2008). Ferritin has also been detected in the unicellular red alga *Cyanidium caldarium* (Nagasaka et al., 2002). Meanwhile, it is suggested that another mechanism for iron storage exists in the unicellular brown alga *Ectocarpus siliculosus*, which has no ortholog of ferritin in its genome (Bottger et al., 2012).

There is a growing demand for recombinant proteins for therapeutic applications. Therapeutic proteins provide important therapies for a variety of diseases, such as diabetes, cancer, infectious diseases, based on these information's our present study has designed to develop the recombinant protein ferritin from marine algae *P. yezoensis* for therapeutic applications.

Here, we investigated the biological activity of ferritin from the marine macroalgae *P. yezoensis* through recombinant DNA technology. High iron levels can affect the expression of genes which encode ferritins (Torti and Torti, 2002), oxidative stress (Tsuji, 2005) and heavy metal ions (Zhang et al., 2006). Furthermore, it has been proved that the recombinant ferritin (CsFerM) obtained from *Cynoglossus semilaevis* has entirely inhibited the six different bacterial fish pathogens (Wang et al., 2011).

Based on these reports, we designed our present study to investigate the antimicrobial and antioxidant activity of recombinant ferritin from *P. yezoensis*. The recombinant ferritin (rPyFer) was expressed in *E. coli* BL21, purified and characterized using various analytical methods, and the purified rPyFer assayed for ferroxidase activity as well as for antibacterial and antioxidant activities. From literature review it has been noted that the Equine spleen ferritin (ESF) is able to catalyze ferrous irons oxidation (ferroxidase activity) and it used for reference standard because of its structural and functional properties (Lakshmi Deepa et al., 2008), based on these information all experiments were performed for ESF and both the results were compared.

Materials and methods

Chemicals

The *P. yezoensis* ferritin gene (PyFer) GenBank (accession no. JX293834) cloned using pET21d was obtained from Bioneer Corporation (Daejeon, South Korea). The expression host *E. coli* BL21 (DE3) was purchased from Enzygnomics (Daejeon, South Korea). ESF was obtained from Sigma-Aldrich (St. Louis, MO, USA). The reagents used for the recombinant expression of PyFer were ampicillin, isopropyl- β -D-thiogalactoside (IPTG), *ferrozine*, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and glutathione (GSH), purchased from Sigma-Aldrich. The analytical grade of other chemicals and reagents were used in this experiment.

Cloning of *P. yezoensis* ferritin cDNA (PyFer)

The PyFer gene (accession no. JX293834) cloned in pET21d was purchased from Bioneer Corporation (Daejeon, South Korea). They cloned the sequence using the primer set (5'-CGTCCTTACCATGACGATG-3' and 5'-CCAGAACTGACATGGGAG-3') into pET21d Expression vector (Novagen, San Diego, CA, USA).

The theoretical isoelectric point (pI) and molecular weight (MW) of the protein were calculated from the deduced amino-acid sequence of PyFer using the pI/MW tool (<http://au.expasy.org/tools/>) (Artimo et al., 2012).

Transformation of plasmids into *E. coli* BL21

High-efficiency transformation was carried out by heat shock method (Froger and Hall, 2007). The pET21d plasmid containing the PyFer gene (ampicillin-resistant) was transferred into *E. coli* BL21 (DE3), and 50 μ g/mL ampicillin was used as the selective antibiotic. Glycerol stocks of transformed cells were stored at -80°C .

Recombinant protein expression

A single colony of *E. coli* BL21 (DE3) containing the gene PyFer was cultured in 10 mL of Luria-Bertani (LB) broth containing ampicillin (50 μ g/mL), then it was incubated overnight at 30°C with shaking at 250 rpm, then reinoculated into a 1-L LB broth with ampicillin and incubated again at 30°C and 250 rpm until the optical density (OD) at 600 nm reached 0.6–0.8. IPTG used to induce the protein expression (0.1 mM final concentration of IPTG from the stock of 1 M). Protein expression was performed for 4 h at 30°C , 250 rpm. After expression centrifugation was

performed to harvest the cell pellets at 12,000 g for 20 min at 4 °C (Studier et al., 1990; Hansen et al., 1998).

Purification of rPyFer

The collected cell pellet was resuspended in phosphate buffered saline containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and sonicated by using the Sonics vibra cell VCX 500 sonicator with 4 pulses of 10 s each giving a pause of 10 s in between each pulse and always keeping the sample on ice. The sonicated slurry was centrifuged at 12000 rpm at 4 °C for 20 min.

The soluble protein was collected as supernatant, then the soluble protein rPyFer was precipitated by using ammonium sulfate (50% saturation) and the ammonium sulfate was removed by dialysis then the purification was performed by anion exchange chromatography using Q-Sepharose (GE-healthcare, Piscataway, NJ, USA), the column was first equilibrated with lysis buffer, and 50 mM Tris- HCL and 50 mM NaCl (pH 7.5) used as the mobile phase, 20 fractions were collected at 1 ml/min flow rate, all fractions were analyzed at 280 nm, peak containing fractions were combined together and size exclusion chromatography was used to separate the rPyFer by FPLC (AKTA Prime Plus, GE healthcare, Piscataway, NJ, USA) using Superdex 200 pg 16/60 column (GE healthcare, Piscataway, NJ, USA). Tris-HCl buffer (50 mM, pH 7.5) was used to pre-equilibrate the column and same buffer used as mobile phase, 0.75 ml/min flow rate and 0.5 MPa pressure was applied and 80 fractions were collected, major peak fractions were concentrated by using Viva ultra-centrifugal device equipped with 10 kDa cut-off membrane. The rPyFer was identified as single band in SDS PAGE. The protein concentration was analyzed using bicinchoninic acid (BCA) assay (Smith et al., 1985) with bovine serum albumin as the protein standard. The molecular weight was determined via 12.5% SDS PAGE.

Characterization of rPyFer

Ultraviolet-visible (UV-vis) spectrophotometry

UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) was used to analyze the ESF and rPyFer with 1-cm path length of quartz cuvette. Corresponding buffers were used as references for the measurements.

Circular dichroism (CD) spectrophotometry

CD spectra of rPyFer and ESF were acquired with a JASCO J-715 spectropolarimeter (JASCO Corp., Tokyo, Japan). Deep UV (240–190 nm) spectra were obtained

with 0.1-cm path length quartz cuvettes. All measurements were taken at least three times to ensure reproducibility. The spectra were analyzed using DICHROWEB and fitted to four structural parameters: α -helix, β -sheet, β -turn, and random coil.

Ferroxidase assay

The ferroxidase activities of purified rPyFer and ESF were determined using a previously described method (Levi et al., 1988). The ferric iron acceptor was apotransferrin and bovine serum albumin (BSA) was used as the control. 0.1 M ferrous ammonium sulfate, 4 mg/mL apotransferrin, 0.2 M sodium acetate (pH 7.0), and 20 μ g/mL rPyFer was used in the reaction mixture, after the reaction, the OD at 470 nm was recorded for 20 min, the reaction mixture without proteins served as blank.

Antibacterial activity of rPyFer: minimum inhibitory concentration (MIC)

The antibacterial activities of rPyFer and ESF was analyzed by Minimum inhibitory concentration (MIC) method (Andrews, 2001), against Gram-positive and Gram-negative bacterial cultures, the following bacterial cultures were purchased from the Korean Collection for Type Cultures (KCTC): *Staphylococcus aureus* (KCTC 1621), *E. coli* (KCTC 2571), and *Bacillus subtilis* (KCTC 1028), *Pseudomonas aeruginosa* (KCTC 1750). Briefly MIC of the rPyFer and ESF was determined by broth dilution method against the mentioned bacterial cultures using 96 well plates (SPL, Pyeongtaek, South Korea), the bacterial cultures were collected at log-phase and they were diluted in Mueller–Hinton broth (MHB) and cultured in 96 well plate at 1×10^5 CFU/mL final concentration, the rPyFer and ESF were prepared at different concentrations such as 3.125, 6.25, 12.5, 25, 50 and 100 μ g/mL they were added to the microplate which is contain already added bacterial cultures, the microplate was incubated for 18 h at 37 °C, and the absorbance was measured at 600 nm by using a FilterMax. F5 Multi-Mode Microplate Reader (Molecular Devices, USA), chloramphenicol was used as the positive control.

Antioxidant activity of rPyFer

DPPH assay

The radical scavenging activity of rPyFer was investigated using 1,1-diphenyl-2-picryl hydrazyl (DPPH) (Burits and Bucar, 2000). Briefly, a 0.1 mM DPPH was prepared by using ethanol. This DPPH (50 μ L) was mixed with 150 μ L of rPyFer at various concentrations such as 5, 10, 15, 20,

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1
1 CTTGAAGGCTGCTGCGGGCGCGTGCTCCGACGTCCGTCTGCGGTCTCTGCCAACCGT
1
61 GGACGCAACCCTCTCTCAACCGACGGCTCCATCTCACCCATCTCTCATCACCCACCTCTC
1 M T M A A F A
121 CGAAGTAGCCGTGTGCCCCCTCCTTCCCTCCGTCCTTACCATGACGATGGCTGCTTTTGC
8 S V A P V G V S T F A P G A S L S T G R
181 CTCCGTTGCCCTGTTGGTGTCTCGACCTTTGCGCCTGGTGCCTTGTCAACCGGTGC
28 P G A G A V A A P S T S R S A A R M T F
241 CCGCGGCGCCGCGCGCGGTGGCGCTCCCTCCACGTCCCGCTCGGCGGCGCGTATGACGTT
48 S S G S P S G G E T I D F S D V D V T D
301 TTCGTCGGGCAGCCCGTCCGGTGGTGAGACGATTGATTCTCCGATGTGGACGTCACCGA
68 A G A Q F S G M V F T P D T A D A P L S
361 CGCTGGTGCCAGTTCTCCGGCATGGTCTTACCCCCGACACCGCCGACGCACCGCTGTC
88 R A N V G F S Q A C Q D A V N N Q I Q V
421 GCGCGCCAACGTTGGTTTCTCGAAGCGTGCCAGGATGCGGTGAACAACAGATCCAGGT
108 E Y T A S Y A Y H A M F A Y F N R D T V
481 GGAGTACACGGCCAGCTATGCTTACCACGCGATGTTTGCTACTTCAACCGTGACACCGT
128 A L P G F A K Y F E E Q S L E E R T H A
541 TGCCCTGCCGGGCTTCGCCAAGTACTTTGAGGAGCAAAGCCTGGAGGAGCGCACGCACGC
148 D E F M R Y M N K R G G Q V V L K P L A
601 GGATGAGTTCATGCGGTATATGAACAAGCGTGCGGGCAGGTGGTGCTCAAGCCGCTGGC
168 V P S M S F N N T D G T S D A V Y A M D
661 AGTGCCAAGCATGAGCTTCAACAACACGGACGGTACGTGCGACGCAGTCTACGCGATGGA
188 L H L Q L E K F V W A K L E E V A A A A
721 TCTTCACTGCAGCTGGAGAAGTTCGTGTGGGCCAAGCTGGAAGAGGTTGCCGCCGCCGC
208 N A D N D L S L A D L I D D Y V Q E Q V
781 CAACGCGGACAACGACTTGAGTCTGGCGGACCTCATAGACGACTACGTACAGGAGCAAGT
228 Q A V K K A A D M V A Q L K R V G T P H
841 CCAGGCGGTCAAGAAGGCGGCCGACATGGTGGCGCAGCTCAAGCGTGTGGGCACCCGCA
248 G V W H F D Q E V L G G E D A Q A *
901 CGGTGTTTGGCACTTTGATCAAGAGGTGCTGGGTGGGGAGGATGCCAGGCGTAATGATG
266
961 CGGCCGTCTCCCATGTGAGTTTCTGGTTTTGTTGATGGATGGATGTGTTGGGCGGAAGTC
275
1021 TCGGGCCGATTGAACGTGGTCCAGGGCCGCAAGGTGCCCGGCAAGAGCACGGGGATGCG
281
1081 CCGCGTGTGGTGAACCGGACTGACCTCTGGCGAGGCCATGTTGCCCTTGCCACTCGGCT
293
1141 CCGAGCTCTTGCCGTGGTTGTGCACTCGTGTGGTTGCAGCCGTAGGCGCGTCGGTCAGT
308
1201 TGGACTTTCTTCGCGGTGCTGCCGTTTGCCTCATGATGGGGTGACGGGGTGCTGTTTGCT
308
1261 TTTTGTGTTGTTGGTGTCTTTTCTGTGTAGGCCGCTGTTGCCTTTTGCAGTGCAAATAGG
308
1321 TTGTCGTGCGCATTGTTGAACCCATTCGGTGACTGGGGTTAGAAGAAGCTCATCACACTT
308
1381 GTGATTGTTGCCACAAGGATGTTTGAATGTGACTCTTGGCTTCTTTTGAAAAAAAAAAAA
308
1441 AAAAAAAAAAAAAA

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Fig. 1 Nucleotide sequence and deduced amino-acid sequence of rPyFer. The signal peptides are shaded gray

Fig. 2 12.5% SDS PAGE. The recombinant protein ferritin expression and purification was analyzed in 12.5% polyacrylamide gel and stained with coomassie brilliant blue R-250, 10 μ g protein sample loaded in each well (A) 1: molecular weight marker; 2: uninduced sample; 3: crude Protein; 4: ammonium sulfate precipitated recombinant protein (rPyFer). 5: Purified rPyFer approximate molecular weight 35–25 kDa. (B) 1: molecular weight marker; 2: equine spleen ferritin 3: Purified rPyFer its molecular weight was approximately 35–25 kDa

Fig. 3 (A) Ultraviolet–visible (UV–vis) spectra and (B) circular dichroism (CD) spectra of rPyFer and Equine spleen ferritin (ESF)

25, and 30 μ g/mL. The mixture was incubated at room temperature for 60 min in the dark, then the absorbance was recorded at 517 nm using UV–Vis spectrophotometer (Shimadzu). The results were compared with the antioxidant activity of ESF; GSH was used as the standard. The DPPH scavenging percentage was calculated using the following equation: DPPH scavenging effect (%) or percent inhibition = $(A_0 - A_1)/A_0 \times 100$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the test or standard sample.

ABTS assay

ABTS assay was performed as previously described (Re et al., 1999). ABTS 7 mM and potassium persulfate 2.4 mM were prepared as stock solutions separately, then they were combined together in equal volumes, the mixture was allowed to stand for 12–16 h in the dark for reaction.

After incubation, the mixed ABTS solution was diluted to obtain an absorbance of 0.700 ± 0.03 at 734 nm using methanol. This ABTS solution (2 mL) was mixed with various concentrations of rPyFer (100 μ L; 5, 10, 15, 20, 25 and 30 μ g/mL) and incubated for 1 min at room temperature and the absorbance was recorded at 734 nm. GSH was used as the standard.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was analyzed in accordance with a previously described method (Benzie and Strain, 1996). Stock solutions of 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$) (pH 3.6), 10 mM 2, 4, 6-tri-pyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution were prepared. Fresh working solution FRAP was prepared by mixing (10:1:1) 25 mL of acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL

Table 1 Minimum inhibitory concentration (MIC) of rPyFer against bacterial cultures

Samples and antibiotic	MIC ($\mu\text{g/mL}$)			
	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>
rPyFer	6.25	12.5	3.12	3.12
ESF	12.5	50	50	50
Chloramphenicol	3.12	3.12	3.12	3.12

Different concentrations of rPyFer and ESF (3.125, 6.25, 12.5, 25, 50 and 100 $\mu\text{g/mL}$) were evaluated against both Gram-negative and Gram-positive bacteria cultures using chloramphenicol as a positive control, triplicate results were recorded

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and then the mixture solution was warmed to 37 °C before use. This FRAP solution 200 μL was mixed with 40 μL rPyFer at various concentrations (5, 10, 15, 20, 25 and 30 $\mu\text{g/mL}$) and incubated for 10 min at room temperature and the absorbance was recorded at 593 nm. GSH was used as the standard.

Statistical analysis

All results were calculated using OriginPro v.8 software (OriginLab Corporation, Northampton, MA, USA) and are expressed as the mean $\pm$ standard deviation.

Fig. 4 Time-course analysis of ferroxidase activity of rPyFer compared to that of bovine serum albumin (BSA) and ESF. The formation of the iron (III)-transferrin complex was monitored at 470 nm. The each point represents the average of 3 experiments, and that error bars indicate the standard deviation

Results and discussion

cDNA cloning of PyFer

The open reading frame of PyFer cDNA comprised 795 bp encoding 264 amino acid. The nucleotide sequence and deduced amino-acid sequence are presented in Fig. 1. The calculated pI and MW were 4.52 and 28.14 kDa, respectively. A number of bacterial and vertebrate ferritins have been expressed in *E. coli* (Levi et al., 1989), but there are fewer studies on ferritin from marine algae, particularly from the red algae *P. yezoensis*. In the present study, the iron-binding protein PyFer was produced using recombinant technology, and the MW of the recombinant protein was $\sim$ 28.14 kDa.

rPyFer expression and purification

Recombinant proteins tend to form inclusion bodies when they are overexpressed in prokaryotic expression systems. The temperature, concentration of selective antibiotics, and inducer concentration are the major factors for improving the solubility of recombinant proteins. In our study, rPyFer from the marine alga *P. yezoensis* was expressed in chemically competent *E. coli* BL21 (DE3), which allows induction by IPTG. After 24 h of incubation at 30 °C, the selection plates contained transformed colonies at a concentration of $\sim 1.5 \times 10^7$ CFU/mL, indicating a successful high-efficiency transformation.

After expression and extraction of the recombinant protein, the obtained soluble protein was further purified, and a single visible protein band with approximate molecular weight between 35 and 25 kDa was found via SDS-PAGE (Fig. 2A), the rPyFer was compared with ESF shown in (Fig. 2B). The ESF showed multiple protein bands from 245 to 10 kDa, a strong protein band occurred approximately 10–15 kDa which represents the rPyFer and ESF is not similar in molecular weight but both are having similar iron binding property. The final concentration of protein was about $\sim$ 1 mg/mL estimated by BCA assay; a total of 5 mg of recombinant protein was obtained from

Fig. 5 Radical-scavenging activity of rPyFer and ESF. DPPH, ABTS and FRAP activities were measured as described in “Materials and methods” section, that each column represents the average of 3 experiments, and that error bars indicate the standard deviation.

1 L of culture medium. The commercial protein ESF concentration was diluted into ~ 1 mg/mL it was confirmed by BCA assay.

Results compared with, glutathione (GSH). (A) 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, (B) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity and the (C) ferric reducing antioxidant power (FRAP) assay

Characterization of rPyFer using various analytical methods

UV-Vis and CD spectrophotometers of rPyFer were performed. Figure 3A shows the UV-Vis spectra produced by rPyFer and ESF; the broad peak observed at ~ 280 nm, similar absorbance at 280 nm was observed in the previous study about UV-Vis spectral analysis of ferritin (May and

Fish, 1978). The CD spectra of rPyFer and ESF represent a mix of helical and β -turn conformations (Fig. 3B). When fitted using DICHROWEB, the following values were obtained for the α -helix, antiparallel β -sheet, and parallel β -sheet: respectively, 72.1% 11.2% and 16.7% and for ESF the values are 70.9% 12.0% and 17.1%, respectively. The CD spectra of rPyFer and ESF were similar.

Antibacterial activity of rPyFer

The antibacterial activity of rPyFer was analyzed using the MIC assay. Overall, rPyFer exhibited highest antibacterial activity against Gram-positive bacterial cultures compared with Gram-negative bacterial cultures. At a concentration of 3.12 $\mu\text{g/mL}$, rPyFer completely inhibited the growth of *S. aureus* and *B. subtilis*, which is similar to the activity exhibited by the standard antibiotic chloramphenicol. Commercial ESF exhibited much lower antimicrobial activity against the tested pathogenic bacterial cultures. The results are represented in Table 1. Ferritin is known to affect bacterial growth, and four purified recombinant protein from *Patinopecten yessoensis* identified that they completely inhibited the growth of pathogens (Zhang et al., 2013). In our present study, rPyFer was able to completely inhibit Gram-positive bacteria at minimal concentrations. The rPyFer is able to bind iron and is thus involved in the innate immune response against microbial infection. Ferritin plays a crucial role in the iron withholding defense system. Iron withholding, as an important innate defense mechanism. Since iron is important for functioning of pathogenic bacteria, deprivation of iron will bring about its destruction (Ong et al., 2006). Thus the mechanism underlying for the antimicrobial action of this rPyFer may be due to the trapping of excess iron molecules in host cells, thereby decreasing plasma iron, which is critical for bacterial growth and pathogenicity. The results were similar to those obtained in a study on turbot, which showed that recombinant ferritin inhibited the growth of pathogens (Zheng et al., 2010).

Ferroxidase activity of rPyFer

The iron-incorporation activity of rPyFer was investigated by the ferroxidase assay. From 5 to 20 min, iron incorporation increased compared to the activity exhibited by the standard protein BSA. ESF also exhibited increased ferroxidase activity compared to rPyFer (Fig. 4). Ferritin plays a crucial role in the metabolism of iron, including the regulation of iron concentration and detoxification (Zhang et al., 2013). The catalytic activity of the ferroxidase enzyme has converted the ferrous ions into ferric ions. The Fe(III)-transferrin complex was formed after the addition of apo-transferrin to rPyFer and ESF it was absorbed at

470 nm. The rPyFer enables the oxidation of Fe^{2+} to Fe^{3+} , with allowing iron to bind with transferrin, and forms a strongly colored chromogenic compound of ferric ions. The chromogen does not make up a complex of ferrous ions in color. Ferrous ions were converted into ferric ions because of the catalytic action of the ferroxidase enzyme (Neselioglu et al., 2017). Ferritin plays a major role in iron metabolism through its ferroxidase activity. Similar result was absorbed in ferroxidase activity of ceruloplasmin (Bakker and Boyer, 1986).

Antioxidant activity

The antioxidant activity of rPyFer was analyzed using DPPH, ABTS and FRAP assays with the standard GSH. The percentage of radical-scavenging was compared with that of ESF (Fig. 5).

Overall, rPyFer exhibited significant antioxidant activity. The scavenging activity of rPyFer was increased in dose dependent manner. In our study, iron uptake and antioxidant experiments with rPyFer revealed significant activity similar to that of ferritins isolated from abalone and clam (Salinas et al., 2011). Altogether, rPyFer from the marine alga *P. yezoensis* acts against microbial pathogens by sequestering host iron and exhibits significant iron-uptake and radical-scavenging activities. In our present study the biological activities of rPyFer was compared with reference standard ESF, the rPyFer showed higher antibacterial activity than the ESF. From the results it has been concluded that the recombinant protein ferritin could be used for the treatment of bacterial infections and its plays a major role in antioxidant activity and ferritin act as an iron storage device in macro algae *P. yezoensis*. Based on this primary investigation, the protein will be further analyzed for its structural and functional characteristics.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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