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Development and fabrication of disease resistance protein in recombinant *Escherichia coli*

Sefli Sri Wahyu Effendi[1†], Shih-I Tan[1†], Chien-Hsiang Chang[1], Chun-Yen Chen[2], Jo-Shu Chang[1,3,4] and I-Son Ng[1*]

Abstract

Cyanobacteria and *Spirulina* produce C-phycocyanin (CPC), a water soluble protein associated pigment, which is extensively used in food and pharmaceutical industries. Other therapeutic proteins might exist in microalgal cells, of which there is limited knowledge. Such proteins/peptides with antibiotic properties are crucial due to the emergence of multi-drug resistant pathogens. In addition, the native expression levels of such disease resistant proteins are low, hindering further investigation. Thus, screening and overexpression of such novel proteins is urgent and important. In this study, a protein which was identified as a putative disease resistance protein (DRP) in the mixture of *Spirulina* product has been explored for the first time. To improve protein expression, DRP was cloned in the pET system, co-transformed with pRARE plasmid for codon optimization and was significantly overexpressed in *E. coli* BL21(DE3) under induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG). Furthermore, soluble DRP exhibited intense antimicrobial activity against predominant pathogens, and an inhibition zone of 1.59 to 1.74 cm was obtained for *E. coli*. At a concentration 4 mg/mL, DRP significantly elevated the growth of *L. rhamnosus* ZY up to twofold showing probable prebiotic activities. Moreover, DRP showed potential as an effective antioxidant, and the scavenging ability for ROS was in the order of hydroxyl > DPPH > superoxide radicals. A putative disease resistance protein (DRP) has been identified, sequenced, cloned and over-expressed in *E. coli* as a functional protein. Thus expressed DRP showed potential anti-microbial and antioxidant properties, with promising therapeutic applications.

Keywords: Disease resistance protein, Recombinant technology, Rare codon, Antibacterial, Antioxidant

Introduction

Microalgae, including diatoms of Bacillariophyta, green algae *Chlorella* sp. and blue-green algae cyanobacteria, serve as a natural carbon sink, and are known as a sustainable feedstock for biodiesel and biofuel production. The protein rich microalgal biomass is also known for the co-production of a number of high-value products viz., carbohydrates, bioplastic polymers, cosmetics, and food additives (Li et al. 2018; Allen et al. 2018). Microalgae and cyanobacteria are naturally protein-rich (Teuling et al. 2019), and C-phycocyanin (CPC) is the dominant phycobiliprotein commonly seen in cyanobacteria (Eriksen 2008). CPC has been explored in pharmaceuticals as antibacterial, anticancer, antioxidants, health supplements, and vitamins mainly due to the increasing demand for alternative antimicrobial agents to counteract the rising antibiotic resistance in pathogens (Singh et al. 2011; Waghmare et al. 2016). The presence of carotenoids and chlorophylls alongside CPC is the major bottleneck in the CPC purification process. Thus the development of a pigment extraction cascade without any loss in essential proteins is vital (Marzorati et al. 2020). Other than that,

*Correspondence: yswu@mail.ncku.edu.tw
†Sefli Sri Wahyu Effendi and Shih-I Tan contributed equally to this work
[1] Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan
Full list of author information is available at the end of the article

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extraction cascades have also been successfully applied to isolate fatty acids from the spent biomass after CPC extraction, which consisted of high amounts of PUFAs (Imbimbo et al. 2019). Furthermore, microalgae and cyanobacteria might possibly contain other therapeutic proteins/peptides with applications as novel drugs. However, discovery, extraction and purification of such novel algal proteins in a sustainable way is a critical issue.

Tandem mass spectrometry serves as a powerful tool to identify proteins and studying the relationship between protein functions and cellular behavior. Protein discovery related to a specific function is essential for advancement in emerging technologies such as synthetic biology which helps in solving global issues (Coon et al. 2005). For instance, the presence of asiaticoside in ethyl acetate extract from medicinal plant *Centella asiatica* Urban was confirmed by LC–MS (Gupta et al. 2018). Therefore, the advent of high-throughput tandem mass spectrometry invigorated proteomics—the classification of the protein complement expressed by the genome of an organism (Wolters et al. 2001). The most common application of proteomics is in the medical sector for therapeutics and diagnosis by identifying novel biomarkers of disease. Recently, Marchand and his colleagues developed a non-natural amino acid system in *E. coli* which was integrated by proteomics (Marchand et al. 2019). Moreover, proteomics plays a vital role in revealing the metabolism under different physiological stimulation and discovering new proteins for unique applications. For example, a robust multiple copper oxidase from an electrogen *Proteus Hauseri* ZMD44 was discovered and overexpressed in *E. coli* to be applied in gold recovery, because the organism exhibited tolerance to copper ions by automatically overexpressing the multiple copper oxidase (Ng et al. 2016; Tan et al. 2017).

Recombinant technology has become an essential tool at hand for high-level production of heterologous proteins (Rosano et al. 2014). The choice of the host cell and it's protein synthesizing machinery is decisive in initiating the outline of the whole process. As a model organism, *E. coli* has been routinely used to produce heterologous proteins (Schlegel et al. 2017). Also, the advantages of using *E. coli* over other organisms are well-known, such as doubling time in glucose-salts media is about 20 min, and it is able to reach high cell density easily and stationary phase can be attained in a few hours (Sezonov et al. 2007). Previous studies have reported that CPC from cyanobacteria was successfully expressed in *E. coli* (Zhao et al. 2006; Guan et al. 2007; Yu et al. 2016).

In this study, a putative disease resistance protein (DRP) was screened from the mixture of *Spirulina* product and identified by MS/MS for the first time. Afterward, the whole DNA sequence of DRP was synthesized, cloned into pET21a(+) and further expressed in *E. coli*. The optimization of DRP production involved optimal codon usage and temperature effect. Furthermore, the novel functionalities of DRP are explored regarding antimicrobial activity, prebiotic promoting activity and antioxidant activity.

Materials and methods

Protein identification from nature product

The natural product, which was initially extracted from *Spirulina* species, was purchased from Febico Bio-Tec, Taiwan. The powder was dissolved in deionized water to a concentration of 10 g/L, and then the solution was centrifuged to collect the supernatant. The protein concentration of the supernatant was measured by Bradford assay (Bio-Rad, USA) with bovine serum albumin (BSA) as a protein standard. The concentration of the protein solution was then adjusted to 1 g/L. The final solution was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the protein pattern, which was visualized by staining with Coomassie blue R-250. The targeted protein bands were sent for tandem MS/MS analysis.

Synthesis and cloning of DRP and co-transformation with pRARE plasmid

After identifying the amino acid sequence by MS/MS analysis, the DNA sequence of DRP was deduced by reverse translation using Vector NTI (Life Technologies, USA) as shown in Additional file 1: Figure S1, and then the entire gene sequence was synthesized by IDT (Coralville, USA). The DRP fragment was amplified by polymerase chain reaction and cloned into pET21a(+) plasmid at the restriction sites *Nde*I and *Xho*I (NEB, USA) (Additional file 1: Figure S2). The strains, plasmids, and primers used in this study are listed in Table 1.

Culture conditions and overexpression of recombinant DRP

The Disease resistance protein (DRP) was cloned and expressed in *E. coli* BL21(DE3). First, recombinant colonies were grown on LB plates (1.5% tryptone, 1.5% NaCl and 0.5% yeast extract) with antibiotics (50 mg/L ampicillin for pET21a(+)-DRP and 12.5 mg/L chloramphenicol for pRARE) at 37 °C for 12 h. Next, a single colony was inoculated in LB medium with appropriate antibiotics for pre-culture at 37 °C for 12 h with shaking at 200 rpm. Then, the cells were diluted 1:100 in LB medium with antibiotics and cultured for about 3 h. Growth was monitored by measuring the biomass or optical density at 600 nm (OD_{600}) using the spectrophotometer (SpectraMax 340, Molecular Devices, USA). As the OD_{600} reached $0.6 \sim 0.8$, the cells were induced by the addition

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Table 1 Strains, plasmids and primers used in this study

Name	Description	Remark
E. coli strains		
DH5α	F-, Δ(argF-lac)169, φ80dlacZ58(M15), ΔphoA8, glnX44(AS), λ-, deoR481, rfbC1, gyrA96(NalR), recA1, endA1, thiE1, hsdR17	Molecular cloning
BL21(DE3)	F^- *ompT gal dcm lon hsdS$_B$(r$_B^-$m$_B^-$)* λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB$^+$*]$_{K-12}$(λ^S)	T7-based expression
Plasmids		
pET21a	3802 bp, Km^R, RSF ori, T7 promoter, T7 terminator, LacI	This study
pET21a-DRP	2922 bp, Cm^R, pUC ori, PlacI promoter, B0034 RBS, sfGFP	This study
pRARE	2935 bp, Cm^R, pUC ori, PI promoter, B0034 RBS, sfGFP	Tegel et al. 2010
Primers		
NdeI-DRP-F	GC<u>CATATG</u>TTGAAGGACGAATTGC	This study
XhoI-DRP-R	TG<u>CTCGAG</u>TCATTAATGGTGGTGATGGTGGTGGCTACCTCTTGG	This study

Restriction sites have been underlined

of 0.1 mM IPTG and further incubated at 25 and 30 °C for up to 12 h. Finally, the cells were harvested by centrifuging at $12,000 \times g$ for 10 min and washed with deionized water for two times. Then, the OD was adjusted to an appropriate concentration, and the cells were disrupted using a One-Shot high-pressure crusher to obtain soluble DRP. The whole-cell proteins and the soluble DRP protein were analyzed by SDS-PAGE.

Antibacterial activity

Antibacterial activity determination was performed by the agar well diffusion method, which was seeded with pathogen strains (Rani et al. 2018). *Aeromonas hydrophila, Bacillus cereus, Escherichia coli,* and *Staphylococcus aureus* were inoculated in 20 mL of LB agar at 2% (*v/v*). For testing the antibacterial activity, various concentrations of soluble protein DRP were absorbed on the filter paper (diameter ± 0.25 cm) in a cooled agar plate. The size of the bacterial inhibition zone for each concentration was measured after culturing at 37 °C for 6 h. The positive control was CPC from natural product purchased from Febico Bio-Tec, Taiwan. All experiments were performed in duplicate independently.

Prebiotic activity

The prebiotic activity test was carried out by culturing 5% (*v/v*) of *Lactobacillus rhamnosus* ZY on 20 mL of MRS medium in a 100 mL of Erlenmeyer flask (Lai et al. 2019). Each culture flask was supplemented with different amounts of BL21(DE3) wild type and the recombinant strain expressing DRP. The cells were cultured at 37 °C with shaking at 200 rpm for 12 h. After that, samples were taken and diluted with deionized water 10^7 times before the screening process on MRS agar plate. The colony-forming units (CFU) of prebiotics were counted after incubation at 37 °C for 24 h.

Antioxidant activity

DRP samples were prepared in different protein concentrations (i.e., $0.1 \sim 4$ mg/mL). Phycocyanin (CPC), one of the functional proteins, was extracted from the commercial product (Febico Bio-Tec, Taiwan) and diluted to 1 mg/mL as a control solution.

DPPH radical scavenging assay

The α, α-diphenyl-β-picrylhydrazil (DPPH) radical scavenging activity was performed according to a previous study (Xia et al. 2011) with some modification. First, 0.039 g of DPPH was added to 1 mL of anhydrous alcohol, and the DPPH solution was diluted 100 times with anhydrous alcohol. A 100 µL of DPPH solution was mixed with 100 µL of protein sample in a centrifuge tube. Samples were incubated in darkness at 25 °C for 30 min. After the reaction was completed, the precipitate was removed by centrifugation at $12,000 \times g$ for 1 min. A control was measured by replacing the protein sample with water. The mixture reaction (A_i) and control (A_0) samples were transferred into 96-well microplate and the absorbance was measured at the wavelength of 517 nm. The DPPH scavenging rate was determined using the following equation:

$$DPPH\ Scavenging\ rate\ (\%) = [1 - (A_i - A_0)/A_i] \times 100\%.$$

OH⁻ radical scavenging assay

The hydroxyl scavenging assay was conducted according to the Fenton reaction (Sies et al. 1993; Wang et al. 2017; Huang et al. 2019) with some modification. First, 0.085 g of $FeSO_4 \cdot 7H_2O$ was added to 50 mL of H_2O_2 to prepare a $FeSO_{4(aq)}$ solution (6 mM). Then, 200 µL of $FeSO_{4(aq)}$, 100 µL of sodium salicylate solution (10 mM), and 20 µL protein sample were added in a centrifuge tube, mixed

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well and incubated for 30 min. The hydroxyl radical reacts with salicylic acid to form 2,3-dihydroxybenzoic acid which absorbs UV light at 510 nm. After the reaction was completed, the precipitate was removed by centrifugation at $2000 \times g$ for 5 min. The reaction mixture was transferred into a 96-well microplate and the absorbance A_i was measured. The background absorbance A_j was measured by replacing the protein sample with water, and the blank absorbance A_0 was measured by replacing the sodium salicylate solution with water. The following formula was used for the calculation of the scavenging rate of hydroxyl radical (OH^-):

$$OH^- \text{ Scavenging rate } (\%) = \left[1 - (A_i - A_0)/A_j\right] \times 100\%.$$

O_2^- radical scavenging assay

The superoxide radical scavenging activity was performed as reported previously (Patel et al. 2018) with some modifications. First, a 10 mM pyrogallol ($C_6H_6O_3$) solution in 10 mM HCl and a 50 mM Tris–HCl buffer at pH 8.2 were prepared. Then, 500 µL of Tris–HCl buffer, 470 µL distilled water, and 10 µL of protein sample were added in a centrifuge tube, mixed well and incubated for 20 min at 25 °C. A 200 µL of the mixture was added to 20 µL of preheated 10 mM pyrogallol solution at 25 °C. The reaction mixture was transferred to a 96-well microplate and the kinetic absorbance (A_i) was measured at the wavelength of 325 nm for 2 min. The oxidation rate of pyrogallol ΔA was estimated by calculating the difference of absorbance per minute in the linear range. The auto-oxidation rate of pyrogallol ΔA_0 was also estimated as above by replacing protein samples with pyrogallol solution. The following formula calculated the scavenging rate of superoxide anion (O_2^-):

$$O_2^- \text{ Scavenging rate } (\%) = (1 - \Delta A/\Delta A_0) \times 100\%.$$

Results and discussion

Identification of disease resistance protein from nature product

The native proteins from the commercial product were analyzed by SDS-PAGE (Fig. 1). The results showed a dominant band at a molecular weight of 17 kDa (NP-17), which was indicated to be the CPC beta subunit with 30% coverage by tandem MS analysis (Table 2). It is reasonable that the dominant band was CPC, because *Spirulina* species are known as a rich source of bioactive products, one of which was CPC (Demay et al. 2019). Besides, CPC has several functional properties such as anti-oxidative function, anti-inflammatory activity, anti-cancer function, immune enhancement function, liver, and kidney
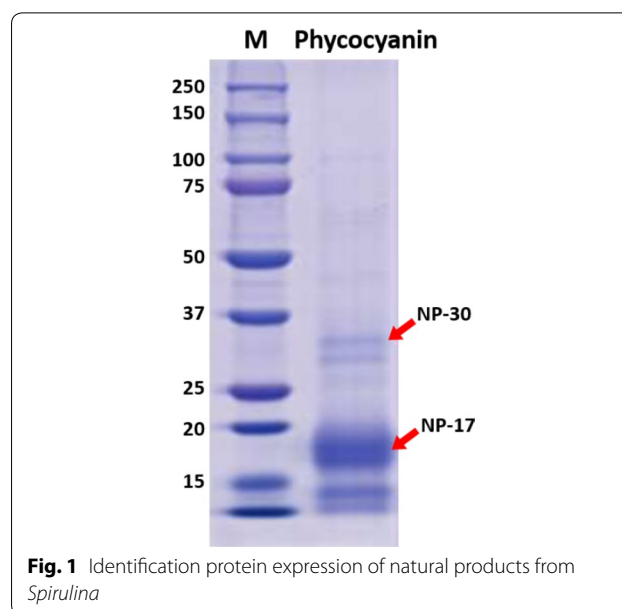
Fig. 1 Identification protein expression of natural products from *Spirulina*

protection and other pharmacological effects (Jiang et al. 2017).

Another protein at a molecular weight of 30 kDa (NP-30) was observed, which was further identified as a putative disease resistance protein (DRP) (Table 2). Interestingly, the homologous protein of DRP was present in a higher plant, *Dichanthelium oligosanthes*, according to the OEL30137 sequence in the GenBank database. We considered that DRP might be present in the natural product purchased from Febico Bio-Tec. On the other hand, the coverage of DRP from MS/MS result was only 5%, because it was present in trace amounts in the natural product, and has never been reported previously. Consequently, the whole DNA sequence for DRP was synthesized artificially and cloned into the pET21a(+) plasmid for overexpression in *E. coli*. The functional properties of the over-expressed DRP were further explored.

Over-expression of recombinant DRP in *E. coli*

The DRP gene acquired by DNA synthesis was amplified by PCR (Fig. 2a), and cloned in pET21a(+) vector. The recombinant gene expression was driven by T7/lac promoter and overexpressed in *E. coli* BL21 (DE3). Protein expression induced by IPTG was evaluated by SDS PAGE analysis. The recombinant protein was not overexpressed under the experimental conditions with the single plasmid (Fig. 2b).

Next, to enhance the expression level of DRP in *E. coli*, pRARE plasmid was co-transformed with pET21a-DRP as dual plasmids and was cultured at 37 °C for 12 h. The pRARE plasmid was used to improve protein expression as it contains the rare codon encoding tRNAs and
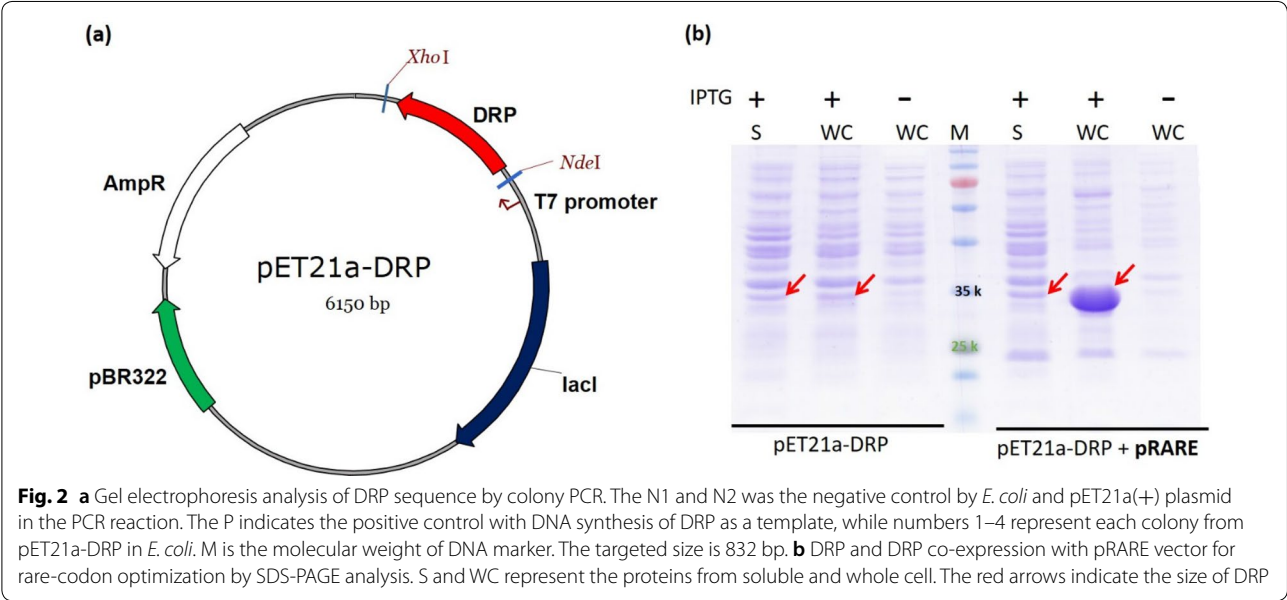
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Table 2 MASCOT analysis of protein identification and full sequence of target protein

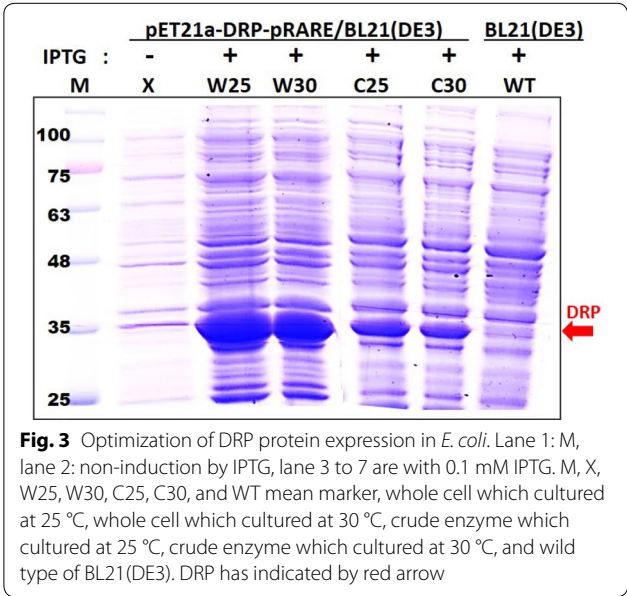| No	Protein name	Accession no.	Score	Mw (kDa)	PI	Coverage (%)
NP-17	C-phycocyanin beta subunit, Chain B	1GH0_B	115	18.0	4.96	30
NP-30	Putative disease resistance protein (DRP), partial	OEL30137	141	27.8	6.20	5

Protein sequence of DRP

MLKDELQRLQGCLKDADTKRRSGNESAAIWVSQIRDAAYEAENVLEVVDHMEKRNRLKKGFMGAVSRYARLPSDLITLHKVGIEIERIRRKVSEISESANRLKILDLGNDELEN-SHLGDESPQDYRLVHQNFEDVTVVVGFEDEQKEIVEELVEKDGKLSVVSIVGMGGAGKTTLARKVYTSDKIKQR<u>FETIAWVTVSQK</u>FKGADLLKDIMKQIMGSEYKPR-QIDQLEEYELGGKIQNFLVQKKNRSLVPRGS

The underlined amino acid sequence is identified by MS/MS and to be the 5% coverage of DRP

Fig. 2 a Gel electrophoresis analysis of DRP sequence by colony PCR. The N1 and N2 was the negative control by *E. coli* and pET21a(+) plasmid in the PCR reaction. The P indicates the positive control with DNA synthesis of DRP as a template, while numbers 1–4 represent each colony from pET21a-DRP in *E. coli*. M is the molecular weight of DNA marker. The targeted size is 832 bp. **b** DRP and DRP co-expression with pRARE vector for rare-codon optimization by SDS-PAGE analysis. S and WC represent the proteins from soluble and whole cell. The red arrows indicate the size of DRP

optimize codon usage for heterologous protein expression (Liu et al. 2006). As expected, the recombinant protein was successfully expressed at approximately 30 kDa; however, most DRP was aggregated into the inclusion body (Fig. 2b). To express the DRP as a soluble protein, the post- IPTG induction temperature was reduced to 30 °C and 25 °C instead of 37 °C. This is because inclusion bodies are formed due to improper folding or conformation of the overexpressed protein at high temperature, and induction at low temperature might assist proper folding of the proteins. This was a common phenomenon when using *E. coli* to produce most of the heterologous protein (Yu et al. 2016). As a result (Fig. 3), it was obvious that the protein expression at lower temperatures was maintained at the same level as that of 37 °C. Also, the soluble protein was significantly enhanced, and the highest soluble DRP was obtained by culturing at 25 °C. Due to the limited knowledge of DRP from commerical *Spirulina* extract, it is hard to evaluate the effect of post-translational modification of DRP at this juncture. In this

Fig. 3 Optimization of DRP protein expression in *E. coli*. Lane 1: M, lane 2: non-induction by IPTG, lane 3 to 7 are with 0.1 mM IPTG. M, X, W25, W30, C25, C30, and WT mean marker, whole cell which cultured at 25 °C, whole cell which cultured at 30 °C, crude enzyme which cultured at 25 °C, crude enzyme which cultured at 30 °C, and wild type of BL21(DE3). DRP has indicated by red arrow

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study, the DRP expressed in *E. coli* is without any post-translational modification. Thus, we need to investigate the functional properties of DRP.
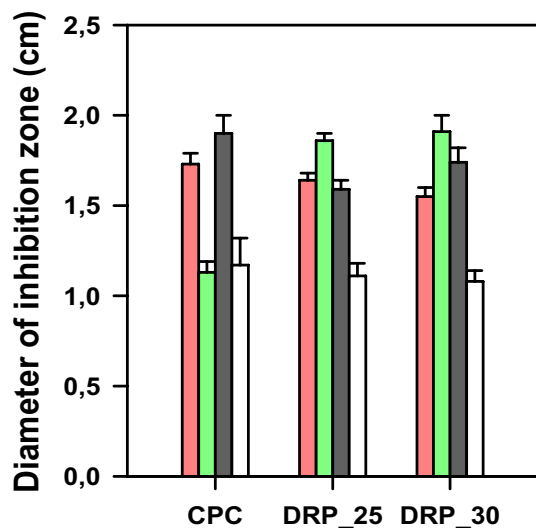
Antibacterial activity of DRP

The recombinant DRP present in the cell-free extract was screened for antimicrobial activity against some relevant pathogens, namely *S. aureus* and *B. cereus* which are Gram positive; *E. coli* and *A. hydrophila* which are Gram-negative (Additional file 1: Table S1, Fig. 4). Among the pathogen strains, *B. cereus* was the most sensitive towards the DRP at 30 °C and 25 °C, with 0.99 ~ 1.91 cm and 1.01 ~ 1.86 cm clearance zone, respectively. Interestingly, *S. aureus* is also Gram-positive, but it was more resistant and showed the minimum clearance zone (0.83 ~ 1.11 cm). The resistance towards CPC was similar for *S. aureus*. In contrast, other studies have reported that *S. aureus* has high sensitivity compared to Gram-negative bacteria towards CPC from exopolysaccharide of *S. thermophilus* GST-6 (Zhang et al. 2016). We considered that the antibacterial compound contained in soluble DRP was slightly different from the one reported by Zhang et al., thus affecting the capability of inhibition in different pathogen strains. Furthermore, 0.25 mg/mL of DRP at 25 °C and 30 °C have shown 1.59 cm and 1.74 cm of clearance zone for *E. coli,* which is a highly virulent pathogen in nature (Silhavy et al. 2010; Najdenski et al. 2013). Besides, extracted commercial CPC has

the strongest inhibition ability for *E. coli* (1.74 cm). Also, the exopolysaccharides from the cyanobacteria *Nostoc commune* (Quan et al. 2015) was highly active against *E. coli*. A high proportion of such antibacterial compound producing strains might be associated with an ecological role, probably displaying defensive measures to maintain their niche, or allow the invasion of strains into established microbial communities (Gillor et al. 2008). On the other hand, both *Spirulina* CPC and the recombinant DRP showed substantial inhibition zones with an increase in protein concentration. The antibacterial activity of DRP at 25 °C and 30 °C is in the order of *B. cereus > E. coli > A. hydrophila > S. aureus.*

Prebiotic activity of DRP

Figure 5 demonstrates the effect of adding different protein supplements on the growth of probiotic strain *L. rhamnosus* ZY. The evaluation was based on the CFU of *L. rhamnosus* ZY screened on MRS agar plate. After 24 h, the CFU of *L. rhamnosus* ZY without protein addition was 6.2×10^8. It is interesting to note that low levels of DRP supplementation successfully promoted the prebiotic activity, even higher than that of CPC at the same concentration (0.5 to 2 mg). Meanwhile, the highest CFU was attained by adding 4 mg/mL of CPC and soluble DRP, and the cell count was elevated up to two-folds (17.5×10^8) and 1.5-folds (16.0×10^8), respectively. Similar to antibacterial activity, the prebiotic activity was concentration-dependent. Despite this, the addition of WT-BL21(DE3) cell free extract seems to be inhibitory,

Fig. 4 Effect of protein added on the antibacterial activities of *Aeromonas hydrophila* (red bar), *Bacillus cereus* (green bar), *Escherichia coli* (grey bar), and *Staphylococcus aureus* (white bar). C-PC is the positive control, while DRP_25 and DRP_30 represent to samples incubator at 25 °C and 30 °C, respectively. All the proteins concentrations are the same as 0.25 mg
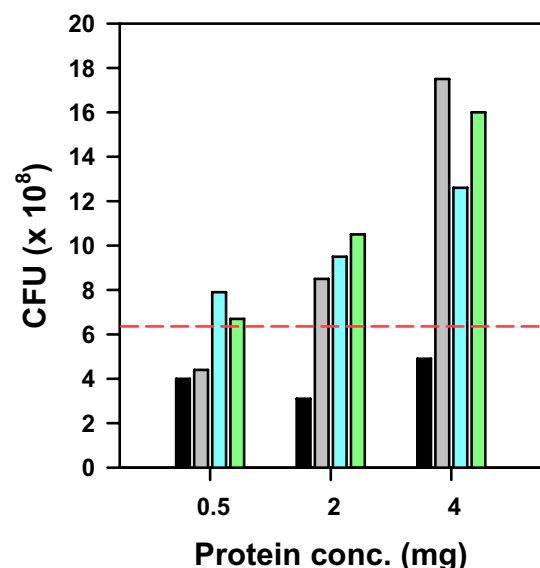

Fig. 5 Effect of protein amounts added from BL21(DE3) (white bar), DRP at 25 °C (black bar), and DRP at 30 °C (grey bar) on the prebiotic activity of *L. rhamnosus* ZY

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since the CFU declined as soluble extract of WT-BL21(DE3) increased. Furthermore, the prebiotic activity of DRP was higher that the proteins obtained from *Chlorella vulgaris* FSP-E and *Chlorella sorokiniana,* since both the algal proteins attained only 7.8 to 8.7×10^8 CFU using a high concentration of protein (Lai et al. 2019).

Antioxidant activity of DRP

The anti-oxidant potential of DRP was assessed by evaluating the scavenging potential of DRP for DPPH, hydroxyl, and superoxide radicals with a wide range of protein concentrations. DPPH is a stable nitrogen-centered free radical substance, and it is commonly used in free-radical scavenging assay (Sánchez-Moreno et al. 2002; Mahmoudi et al. 2020). Stable DPPH exhibits a violet color; and when the DPPH radical accepts an electron from an antioxidant compound, the color of DPPH turns yellow. The degree of discoloration indicates the scavenging potential of the antioxidant extract. Besides, it is most likely a decrease in absorbance caused by phenolic compounds in the reduction reaction between DPPH radicals and antioxidant molecules in protein. The results in Fig. 6a showed that the antioxidant activity of DRP expressed at 30 °C is higher than
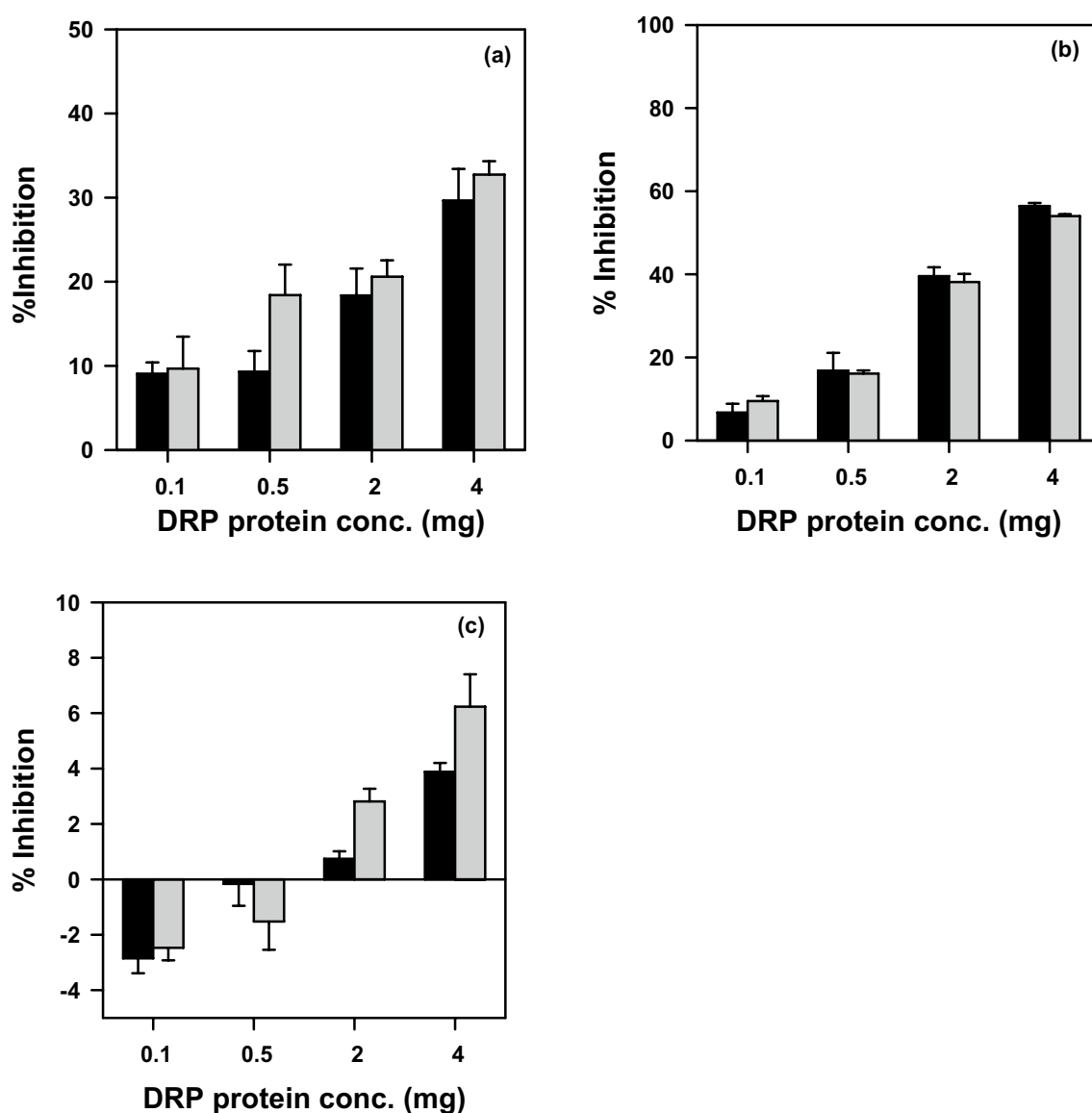
Fig. 6 Functional test based on (**a**) DPPH, (**b**) Hydroxyl, and (c) Superoxide radical scavenging activity of DRP protein, which cultured at 25 °C (black bar) and at 30 °C (grey bar)

that at 25 °C. The antioxidant activity in both the samples improved by increasing the concentration of DRP, and 4 mg/mL showed the highest rate at 32.7% and 29.6%, respectively. The increasing of scavenging activity in protein extract might be due to the presence of antioxidant compounds, which are good electron donors (Easwar and Viswanatha 2020).

Hydroxyl radical is an extremely reactive free radical formed in a biological system. It has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. This radical has the capacity to join nucleotides in DNA and cause strand breakage (Kaur et al. 2019). In this study, the results showed that soluble DRP effectively scavenged the reactive hydroxyl radical, and the antioxidant activity reached 56.4% and 54.0% at 25 °C and 30 °C, respectively (Fig. 6b). The hydroxyl radical scavenging activity of soluble DRP was higher than a previous study, which used *Spirulina* extract (Zayadi et al. 2020).

The superoxide radical scavenging activity results are shown in Fig. 6c. Among various concentrations of soluble DRP, only high concentrations (2 and 4 mg/mL) scavenged superoxide radical about $0.7 \sim 6.2\%$, while soluble DRP at 0.1 and 0.5 mg/mL had no superoxide radical scavenging activity. According to a previous study, the antioxidant activity against superoxide radical might be supported by CPC (Santiago-Morales et al. 2018).

By comparing the antioxidant activities of DRP and CPC from the commercial product as a control (Table 3), the extracted CPC revealed higher anti-oxidant potential than crude DRP at all assessments. Herein, DRP and CPC displayed the highest activity against hydroxyl radicals, which was 90.3% and 56.4%, respectively. The difference between the effect of CPC and DRP on the DPPH scavenging activity was not significant; meanwhile, the superoxide scavenging activity of CPC was tenfolds higher

compared to DRP. However, the protein concentration of DRP was apparently 4 times higher than that of CPC. The results suggested that the scavenging capability was truly influenced by pure phycocyanin, although at less amount. Besides, the antioxidant potential of crude protein from *E. coli* BL21(DE3) was also assessed; however, no antioxidant activity was detected (data not shown).

Furthermore, early studies from varied species exhibited high antioxidant activity. Zhang and his colleagues evaluated antioxidant activity from *Lactobacillus plantarum* C88 (2013) and microalgae strains (2019), including *Chlorococcum* sp., *Scenedemus* sp., and *C. pyrenoidosa* FACHB-9. The results demonstrated that originally microalgae strains displayed higher scavenging ability than *L. plantarum C88* even though the concentration was four times lower, and it successfully scavenged DPPH and OH^- radical about $36.5 \sim 58\%$ and $63.1 \sim 77.5\%$, respectively. It is worth noting that most of the studies cited use a purified product. However, to our best knowledge, purification of the recombinant DRP is laborious and expensive. Recently, researchers have applied some techniques to modify the biological activities of polysaccharides and CPC as well as enhance the antioxidant ability. Box-Behnken design was utilized beneficially to improve the antioxidant potential of microalgae *P. versicolor* NCC466, which showed an anti-oxidant activity of 88.7% for OH^- and 87.4% for O_2^- (Gammoudi et al. 2019). Yu et al. (2016) successfully scavenged the three free radicals (OH^-, DPPH, and O_2^-) by *Synechocystis* PCC6803 by combining Plackett–Burman design and Box-Behnken design which was up to 78, 83, and 64%, respectively.

For a cost-effective process and screening a novel antioxidant agent candidate, this study successfully characterized the antioxidant activity of DRP using cell-free extract protein, which efficiently reduced process costs

Table 3 Comparison antioxidant activity of different protein sources

Protein sources	Antioxidant scavenging activity (%)			Concentration (mg/mL)	Reference
	DPPH	OH^-	O_2^-		
Lactobacillus plantarum C88	52.2	85.2	ND	4	Zhang et al. 2013
Chlorococcum sp.	58.0	77.5	ND	1	Zhang et al. 2019
Scenedemus sp.	51.4	72.4	ND	1	
Chlorella pyrenoidosa FACHB-9	36.5	63.1	ND	1	
Phormidium versicolor NCC466	ND	88.7	87.4	1	Gammoudi et al. 2019
Synechocystis PCC6803	83.0	78.0	64.0	–	Yu et al. 2016
CPC (commercial)	27.4	90.3	30.5	1	This study
DRP_25 °C	29.6	56.4	3.9	4	
DRP_30 °C	32.7	54.0	6.2	4	

ND means not-determined

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due to non-intricated techniques. The crude extract with DRP showed the highest anti-oxidant activity against the hydroxyl radicals. Despite this, purification of the protein is still needed for further examination to guarantee the safety of DRP use. Besides, another viable strategy is to use the probiotic *E. coli* Nissle 1917 for the over-expression of DRP, which produces neither hemolysin nor other toxins.

Conclusion

We hereby demonstrated the process flow of the discovery of a novel protein from natural products and subsequent identification as a disease resistance protein (DRP). The overexpression of DRP was facilitated in the recombinant *E. coli* using pRARE plasmid for codon optimization. The crude extract containing DRP displayed potent inhibitory effects against common bacterial pathogens and showed prebiotic activity on the growth of *L. rhamnosus* ZY cells. DRP also showed strong anti-oxidant activity against hydroxyl radicals. The results reveal that DRP is a powerful candidate for nullifying free radicals.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-020-00343-5.

> **Additional file 1: Figure S1.** The deducded DNA sequence of DRP. **Figure S2**. The gel electrophoresis analysis of the construction of pET21a-DRP by colony PCR. The N1 and the N2 was the negative control by adding the W3110 colony and pET21a plasmid into the PCR reaction. The P indicates the positive control with the addition of DNA synthesis of DRP as a template into the PCR reaction. The number 1 to 4 represents each colony. The targeted size is 832 bp. **Table S1.** Inhibition zone results of DRP protein in different pathogen strains.

Authors' contributions
ISN and SIT designed the experiment and analyzed the data, SSWE and SIT performed all of experiments. ISN, SSWE and SIT wrote the manuscript. CHC, JSC and CYC gave conceptual suggestion. All authors read and approved the final manuscript.

Availability of data and materials
The authors approved the availability of data and materials for publishing the manuscript.

Ethics approval and consent to participate
All the authors have read and agreed the ethics for publishing the manuscript.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

Author details
[1] Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan. [2] University Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan. [3] Department of Chemical and Materials Engineering, College of Engineering, Tunghai University, Taichung, Taiwan. [4] Research Center for Energy Technology and Strategy, National Cheng Kung University, Tainan, Taiwan.

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