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Anti-oxidant activity of holo- and apo-c-phycocyanin and their protective effects on human erythrocytes

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ABSTRACT

This study was conducted to investigate the anti-oxidant activity of the recombinant apo-c-phycocyanin (c-PC) β-subunit compared to native c-PC purified from *Spirulina* sp. The gene encoding the β-subunit of c-PC was successfully cloned and expressed in *Escherichia coli*. The anti-oxidant capacities of recombinant apo-c-PC(β) and native c-PC were evaluated by measuring their Trolox equivalent antioxidant capacities and examining their protective effects on erythrocytes from normal and homozygous haemoglobin E individuals against peroxyl radicals and hydrogen peroxide. The results demonstrated that the anti-oxidant capacities are native c-PC ≫ Trolox > recombinant apo-c-PC(β). Both anti-oxidant proteins can potentially protect erythrocytes from oxidative damage. Expression of c-PC in bacteria reduces the cost and time for protein production, and the recombinant protein could be further developed to obtain a more efficient protein for therapeutic purposes.

1. Introduction

Excess reactive oxygen species (ROS) or loss of the ability to detoxify them results in oxidative stress, which subsequently causes damage to cell components, including DNA, lipids, proteins, and the cell membrane [1]. Oxidative damage has been implicated in the pathogenesis of many diseases, such as Parkinson's disease, Alzheimer's disease, atherosclerosis, cancer, and sickle cell disease [1–3].

c-Phycocyanin (c-PC), a blue pigment found in cyanobacteria and red algae, is well recognised as a potent anti-oxidant [4–6]. It belongs to the light-harvesting phycobiliprotein family, along with allophycocyanin and phycoerythrin [7]. c-PC is usually present as a polymer that is composed of α-and β-subunits. In nature, c-PC is often found as monomer $(\alpha\beta)$, trimer $(\alpha\beta)_3$, or hexamer $(\alpha\beta)_6$. Each subunit of c-PC contains linear tetrapyrrole chromophores, namely phycocyanobilin, which are covalently bound to apo-phycocyanin [8]. In addition to its anti-oxidant activity, various beneficial physiological effects of c-PC have been demonstrated, including anti-cancer [9], anti-inflammatory [10], anti-platelet aggregation [10,11], and anti-hyperalgesic [12] effects.

Recent studies have showed that c-PC from cyanobacteria can be cloned and expressed in *Escherichia coli* [13,14]. Recombinant c-PC retained the absorption and fluorescence characteristics of the native protein. However, the biosynthesis of c-PC in its holo form requires five essential cyanobacterial genes, including *cpcA* or *cpcB*, which encode the apo-phycocyanin α or β subunits, respectively; *cpcE/cpcF* or *cpcS*, which encode phycocyanobilin lyase (used for attachment of phycocyanobilins to c-PC at the correct position); *hox1*, which encodes haem oxygenase; and *pcyA*, which encodes phycocyanobilin:ferredoxin oxidoreductase (used to convert haem to phycocyanobilin). Gaun et al. showed that recombinant holo-c-PC(α) is effective in scavenging hydroxyl and peroxyl radicals [15]. Wang et al. successfully expressed recombinant GST-tagged apo-c-PC(β) from *Anabaena* PCC 7120 in *E. coli*, and the protein was found to inhibit proliferation and induce apoptosis in cancer cells [16]. However, little is known about the anti-oxidant activities of apo-phycocyanin.

Erythrocytes are frequently used as a model for investigating the potential activity of many anti-oxidants. They are the preferential physiological targets of ROS, as their membranes contain a high level of polyunsaturated fatty acids (PUFA). Additionally, they carry high levels of intracellular oxygen and iron, which are important catalysts of oxidation reactions. Erythrocytes that are exposed to excess ROS from either an exogenous or endogenous source undergo cell lysis due to of oxidative damage, including membrane lipid peroxidation, cell deformability, morphological

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alterations, and the cross linking of membrane proteins [17–19]. Certain types of abnormal haemoglobins were found to aggravate oxidative stress in erythrocytes. The most common abnormal haemoglobin in Southeast Asia, haemoglobin E ($\beta 26_{Glu \rightarrow Lys}$), is an oxidatively unstable haemoglobin, which possibly has a weakened alpha 1 beta 1 interaction [20]. The presence of haemoglobin E and excess of α-globin in haemoglobin E homozygotes or β-thalassaemia/HbE patiens results in increased erythrocyte oxidative stress, which overrides the erythrocyte redox capacity, leading to the release of free radicals with consequential severe pathophysiologies [21–23]. There have been reports in β-thalassaemia and β-thalassaemia/HbE patients that treatment with antioxidant substances are shown to reduce erythrocyte oxidative stress [24,25].

In this study, the β-subunit of apo-c-PC from *Spirulina platensis* was cloned and expressed in *E. coli*. Its potential anti-oxidant activity was investigated and compared with those of native c-PC and a standard anti-oxidant, Trolox. Moreover, the effects of apo-c-PC(β) and native c-PC on the protection of erythrocytes of a normal subject and a haemoglobin E homozygote from oxidative damage were explored.

2. Materials and methods

2.1. Chemicals

Phusion *Pfu* DNA polymerase and the protein molecular weight markers were from Thermo Scientific (Waltham, MA, USA). T4 DNA ligase was from Promega (Madison, WI, USA). X-gal and the restriction enzymes were from Fermentas (Vilnius, LTU). The His60 Ni Superflow™ Resin was from Clontech (Mountain View, CA, USA). DEAE-Sepharose Fast Flow was from GE Healthcare (Uppsala, SE). ABTS and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were from Sigma (St. Louis, MO, USA). LB broth, agarose, AAPH [2,2′-azobis-(2-amidinopropane) dihydrochloride], and hydrogen peroxide were from Merck (Darmstadt, DEU). Isopropyl thio-β-D-galactopyranoside (IPTG) was from US Biology (MA, USA). Ampicillin, imidazole, acrylamide, and bis-acrylamide were from Bio Basic Inc (Ontario, CAN). Sodium dodecyl sulphate (SDS), TEMED, and ammonium persulphate were from Bio-Rad (Richmond, CA, USA).

2.2. Organisms and plasmids

S. platensis, the source of the *cpc*B gene, was a gift from Suppamaethakorn W. (Taweewattana Farm, Bangkok, THA). *E. coli* strains DH5α and BL21(DE3)pLysS were used as hosts for DNA manipulation and protein expression, respectively. Plasmid pGEM®-T Easy was obtained from Promega (Madison, WI, USA). Plasmid pETDuet™-1 was purchased from Novagen (Merck, Darmstadt, DEU).

2.3. Cloning and expression of the cpcB gene from S. platensis

The cloning process was carried out using standard procedures according to Sambrook et al. [26]. The genomic DNA harbouring the *cpcB* gene was extracted from *S. platensis* using TRI-reagent (Molecular Research Center, Cincinnati, OH, USA). For gene amplification, a pair of primers was designed according to the *cpcB* gene sequence of *S. platensis* obtained from GenBank (accession no. Y09074). The forward primer was 5′-ACGGATCCACTGGTTCCGCGTGGATCTATGTTTGATGCCTTCAC-3′, and the reverse primer was 5′-GCGAGCTCTTAGGAAACTGCAGCAC-3′. Specific sequences for *Bam*HI and *Sac*I recognition were included (underlined) for cloning purposes. The amplified fragment (554 bp) was subjected to dATP addition at its 3′ end using a dATP attachment kit (Fermentas, Waltham, MA, USA) and then ligated into pGEM®-T, resulting in the plasmid pGem/cpcB, which was transformed into competent *E. coli* DH5α. The transformants were grown for 18 h at 37 °C on Luria-Bertani (LB) agar containing 100 μg/mL ampicillin, 40 mg/mL X-gal, and 0.1 M IPTG. White colonies were selected and screened for the presence of the inserted gene by colony PCR using the *cpcB* primers.

The pGem/cpcB recombinant plasmid was extracted from the positive clone and digested with the restriction enzymes *Bam*HI and *Sac*I to obtain the *cpcB* gene fragment. The fragment was subcloned into the *Bam*HI-*Sac*I site of pETDuet™-1 to create the plasmid pETD/cpcB in which the *cpcB* gene was located downstream of the hexahistidine encoded region. Colony PCR was performed to screen for clones with the gene insertion. The plasmid was extracted and subjected to restriction endonuclease analysis and DNA sequencing for confirmation of the correct gene sequence.

The plasmid pETD/cpcB was then transferred into *E. coli* BL21. To express the hexahistidine-tagged apo-c-PC(β) protein, *E. coli* BL21 harbouring pETD/cpcB was grown at 37 °C until the OD_{600} reached 0.5. The culture temperature was then reduced to 20 °C, and IPTG was added to a final concentration of 0.5 or 1.0 mM. The cells were grown for 12 h and then harvested by centrifugation at 4500 rpm and 4 °C for 10 min. They were lysed by ultrasonication in phosphate buffered saline (50 mM Na_2HPO_4, 300 mM NaCl, pH 7.4) containing 1 mM PMSF using a 5-s pulse on/5-s pulse off programme for 3 cycles. The insoluble and soluble fractions were separated by centrifugation at 12,000 rpm and 4 °C for 20 min and collected for further analysis.

2.4. Purification of the recombinant apo-c-phycocyanin beta subunit and c-phycocyanin from S. platensis

For purification of apo-c-PC(β), the cells were grown, harvested, and lysed according to the expression procedure described above. The supernatant was filtered through a 0.2-μm Millipore filter before loading onto a column containing 10 mL His60 Ni Superflow™ Resin pre-equilibrated with equilibration buffer (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, pH 7.4). The purification was performed according to the manufacturer's recommendation. The apo-c-PC(β) protein was eluted with 10 column volumes of His60 Ni elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 400 mM imidazole, pH 7.4).

Native c-phycocyanin (c-PC) was purified according to Boussiba and Richmond [27] with some modifications. Briefly, 10 g of *S. platensis* cells were suspended in 200 mL phosphate buffer containing 0.1 mg/mL lysozyme and stirred at 30 °C overnight. The cell lysate was centrifuged at 4500 rpm and 4 °C for 20 min, and the blue supernatant containing c-PC was collected. The supernatant was fractionated using ice-cold 35% ammonium sulphate and centrifugation at 10,000 rpm and 4 °C for 20 min. The supernatant was collected, and crude c-PC was precipitated using ice-cold 50% ammonium sulphate. The sample was then centrifuged at 10,000 rpm and 4 °C for 20 min. The blue precipitate containing c-PC was collected and dissolved in 0.025 M sodium phosphate buffer (0.025 M Na_2HPO_4, 0.025 M NaH_2PO_4, pH 7.4) and dialysed against the same buffer to remove the ammonium sulphate. The dialysed solution containing c-PC was further purified using a DEAE-Sepharose Fast Flow column (2.5 cm × 30 cm) according to the manufacturer's recommendations. Stepwise elution was performed using Tris–HCl buffer (pH 7.4) with increasing ionic strength (0–0.25 M NaCl) at a flow rate of 1 mL/min. Two-millilitre fractions were collected and tested for the presence of c-PC by measuring the absorbance at 280 nm and 620 nm. The fractions with an Abs_{620}/Abs_{280} ratio greater than 4.0 were pooled and designated as purified c-PC. The pooled fractions were dialysed against 0.025 M sodium phosphate buffer (pH 7.4) and concentrated to the desired