

Preparation and Antioxidant Activities In Vitro of a Designed Antioxidant Peptide from *Pinctada fucata* by Recombinant *Escherichia coli*

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An antioxidant peptide derived from *Pinctada fucata* meat using an Alcalase2.4L enzymatic hydrolysis method (named AOP) and identified by LC-TOF-MS has promising clinical potential for generating cosmetic products that protect skin from sunshine. To date, there have been few published studies investigating the structure-activity relationship in these peptides. To prepare antioxidant peptides better and improve their stability, the design and expression of an antioxidant peptide from *Pinctada fucata* (named DSAOP) was studied. The peptide contains a common precursor of an expression vector containing an α -helix tandemly linked according to the BamHI restriction sites. The DNA fragments encoding DSAOP were synthesized and subcloned into the expression vector pET-30a (+), and the peptide was expressed mostly as soluble protein in recombinant *Escherichia coli*. Meanwhile, the DPPH radical scavenging activity, superoxide radical scavenging activity, and hydroxyl radical scavenging activity of DSAOP IC₅₀ values were 0.136 ± 0.006 , 0.625 ± 0.025 , and 0.306 ± 0.015 mg/ml, respectively, with 2-fold higher DPPH radical scavenging activity compared with chemosynthesized AOP ($p < 0.05$), as well as higher superoxide radical scavenging activity compared with natural AOP ($p < 0.05$). This preparation method was at the international advanced level. Furthermore, pilot-scale production results showed that DSAOP was expressed successfully in fermenter cultures, which indicated that the design strategy and expression methods would be useful for obtaining substantial amounts of stable peptides at low costs. These results showed that DSAOP produced with recombinant *Escherichia coli* could be useful in cosmetic skin care products, health foods, and pharmaceuticals.

Keywords: Preparation, designed antioxidant peptide, spatial conformation, recombinant *Escherichia coli*

Introduction

Free radicals, such as superoxide anions ($O_2^{\cdot -}$) or hydroxyl radicals ($HO\cdot$), can cause protein damage and DNA mutations that can initiate several diseases, including coronary heart diseases and diabetes [1]. Antioxidants that can scavenge free radicals play an important role in biological systems. Chemically synthesized antioxidants, such as BHT, BHA, and vitamin E, are still widely used in cosmetics and health foods to prevent oxidation. However, the synthesized antioxidants that are used have strict limitations due to

potential toxic effects in the human body. As a result, there is considerable interest in natural antioxidants that can be used to replace chemically synthesized antioxidants in cosmetics or health foods [2]. Recently, food-derived antioxidant oligopeptides have received a large amount of attention because of their good antioxidant activity, safety, easy absorption in the human body, and potential applications as additives in cosmetics or health foods. Antioxidant oligopeptide activity is being investigated in vitro using DPPH radical scavenging activity, superoxide radical scavenging activity, and hydroxyl radical scavenging

activity assays. Examples include antioxidant peptides obtained through the digestion of proteins from animal and plant products, such as egg yolk [3], corn gluten meal [4], egg white protein hydrolysate [5], porcine plasma [6] and Royal jelly [7]. In addition, proteins from marine organisms have also been shown to be a good source of antioxidant peptides, including Pacific hake hydrolysates [8], skipjack tuna meat [9], and silver carp [10]. These food-derived antioxidant peptides have great economic value and application prospects as additives or functional ingredients in cosmetics or health foods.

Pinctada fucata belongs to the family of pearl oysters from the phylum Mollusca. This species is distributed mainly in the Guangxi, Guangdong, and Hainan provinces of China, the Taiwan Strait, and coastal areas of Japan. Currently, with the development of enzyme hydrolysis techniques and purification techniques, increasing numbers of antioxidant peptides from *Pinctada fucata* meat have been prepared and identified by LC-TOF-MS to clarify the relationship between structure and functional activity [11, 12]. To date, preparation methods primarily include chemical synthesis, enzymatic hydrolysis, and expression to produce antioxidant peptides. However, the low rates of enzymatic hydrolysis extraction and purification, as well as the higher costs for purifying antioxidant peptides from *Pinctada fucata* meat, suggest that this natural and safe antioxidant peptide would not meet industrialized production requirements. Currently, most antioxidant peptides are still in the laboratory-scale research stage [13]. Therefore, research on expression methods should be focused on solving the problems associated with preparing high-purity antioxidant peptides. The sequence of antioxidant peptides isolated from *Pinctada fucata* meat typically includes 10 to 20 amino acids, which is relatively short and easily degraded by protease or peptidase in *Escherichia coli* expression systems. Therefore, it is difficult to express these peptides directly. Fortunately, if we can design an antioxidant peptide sequence using biocomputing analysis to enhance expression, this limitation could be resolved. In addition to increasing expression, this strategy may also substantially maintain the natural structure and antioxidant activity compared with chemically synthesized peptides obtained through enzymatic hydrolysis. In this study, we focused on the design and expression of an antioxidant peptide from *Pinctada fucata* meat using recombinant *E. coli*. The corresponding mRNAs of the designed antioxidant peptide sequences were optimized using molecular biology software, and the structure was predicated along with the physicochemical characteristics and spatial conformation through biocomputing analysis

to achieve a high level of expression. The new antioxidant peptide was named DSAOP. Furthermore, DSAOP antioxidant activities were determined in vitro using DPPH radical scavenging, superoxide radical scavenging, and hydroxyl radical scavenging assays. The DSAOP stability and scale-up production in fermentation were also considered. In summary, this protocol established a new strategy to prepare antioxidant peptides with higher purity using an expression system and to overcome the limitations of lower yield or high costs. We are seeking to produce designed antioxidant peptides with high purity using the recombinant *E. coli* expression system and to perform scale-up amplification experiments that could be effective in a broad range of applications in cosmetics and health foods.

Materials and Methods

Materials and Chemicals

Escherichia coli Rosetta (DE3) and the expression pET-30a (+) vectors came from our laboratory. The DSAOP sequence and the primers were chemically synthesized by Sangon (China). The MINIBEST DNA Fragment Purification Kit ver. 4.0 and MINIBEST Plasmid Purification Kit ver. 4.0 were from Takara (Japan). DNA gel extraction kits came from Invitrogen (Thermo Fisher Scientific, USA). Ni-NTA Sefinose Resin was obtained from Sangon. DNA markers of 2,000 bp, T4 DNA ligase, and restriction endonucleases (BamHI and XhoI) were purchased from Takara. Kanamycin, isopropyl- β -D-1-thiogalactopyranoside (IPTG), DPPH, tryptone, and yeast extract were purchased from Solarbio (China). All other chemicals were of analytical grade and purchased from commercial sources. The bicinchoninic acid (BCA) protein assay kit and protein markers were obtained from Thermo Fisher Scientific.

Design of Recombinant Antioxidant Peptides from *Pinctada fucata* Meat

Based on a previous report that an antioxidant peptide from *Pinctada fucata* meat (amino acid sequence was GAGLPGKRER, named AOP) had high antioxidant activity in vitro and potential applications in cosmetics, a design containing the GAGLPGKRER sequence was constructed for further expression to obtain a higher yield and be used in next-step applications. According to Farvin *et al.* [14], certain amino acids site in the antioxidant peptides generated by enzymatic hydrolysis methods might change the secondary structure, which might improve the oligopeptide stability or antioxidant activity of the amino acid residues. Therefore, the amino acid sequence of the designed antioxidant peptide from *Pinctada fucata* meat was MNGGAG LPGKRERNGGAGLPGKRERNGGAGLPGKRERNGGAGLPGKRE RGGAGLPGKRERNG to obtain a stable secondary structure or longer activity time in cosmetic applications in room temperature environment. Meanwhile, the key amino acid site, stable antioxidant

activity, and solubility of the designed polypeptide were considered, the link fragment was chosen, and there was a tandem antioxidant peptide at the N-terminus containing an α -helix structure in the pET30a (+) vector. Therefore, the new amino acid sequence of the designed peptide eventually became MHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDEKAMADIGSMNGGAGLPGKRERNGGAGLPGKRERNGGAGLPGKRERNGGAGLPGKRE RGGAGLPGKRERNG, which was named DSAOP.

Biocomputing Analysis of DSAOP

According to the methods of Axarli *et al.* [15] for bioinformatics analysis, the physicochemical properties of the DSAOP sequence were obtained for further expression and preparation. The programme at the website <http://web.expasy.org/protscale/> provided some predefined scales from the literature that are used for the analysis of structural models. Meanwhile, SWISS-MODEL at the website <https://swissmodel.expasy.org/> could analyze the spatial structure by simulating target protein tertiary structures used for designing the amino acid sequence.

Construction of Recombinant Engineering Bacteria

The DSAOP DNA sequence encoding 113 amino acids was synthesized and codon-optimized for *E. coli* expression using Primer Premier 5.0 [16]. At the end of the design process, the corresponding gene sequence of DSAOP after code-optimization was 5'-ATGCACCATCATCATCATCATTCTCTGGTCTGGTCCGCGGTTCTGGTATGAAAGAAACCGCTGCTGCTAAATTCGAA CGCCAGCACATGGACAGCCAGATCTGGGTACCGACGACGAC GACAAGGCCATGGCTGATATCGGATCCATGAATGGTGGTGCA GGTCTGCCTGGTAAACGTGAGCGTAATGGTGGTGGTGGCCTG CCAGGTAACGTGAACGCAACGGTGGCGCAGGTCTGCCGGGC AAGCGCAACGTAACGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT GAGCGCAACGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT AATGGTTAACTCGAG-3'. The parts marked by italics and underlined denote restriction endonuclease sites. Furthermore, to construct a recombinant vector, the target gene DSAOP and pET30a (+) vectors containing the linker flanked by BamHI and XhoI restriction sites were digested with the corresponding enzymes and ligated with each other. Following ligation, the resulting pET30a (+)-DSAOP (pET-DSAOP) vectors were transformed into chemically competent DE3 cells and plated on agar plates containing kanamycin. Single colonies were screened using restriction digestion and DNA sequencing (Sangon) for successful ligation. Positive clones containing the correct recombinant vector were transformed into competent DE3 cells for subsequent protein expression, and glycerol stocks of the transformed cells were prepared and stored at -80°C . The stocks were named DE3-pET-DSAOP.

Expression of Recombinant DSAOP

Cells carrying the recombinant pET-DSAOP vector (5% (v/v)) were inoculated in sterile Luria-Bertani (LB) medium containing kanamycin (50 $\mu\text{g}/\text{ml}$). Samples were precultured at 37°C in a

rotary shaker (220 rpm) until the OD_{600} reached 0.8–1.0. Next, IPTG was added to a final concentration of 0.5 mM, and the strains of DE3-pET-DSAOP were cultured for an additional 12 h at 20°C , 6 h at 30°C , and 6 h at 37°C to induce production of the target protein. A negative control without IPTG was included. At the end of the induction period, the thallus was harvested using centrifugation at $2,739 \times g$ for 10 min at 4°C and sonicated to dissolve the target protein in the buffer. After centrifugation at $15,777 \times g$ for 20 min at 4°C , the lysates and supernatant were collected and separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining and western blot analysis to determine expression levels.

Purification of Recombinant DSAOP

For DSAOP purification, precultured samples were inoculated (5% (v/v)) in fresh LB broth in the presence of kanamycin (50 $\mu\text{g}/\text{ml}$) at 37°C for 4 h. The cells (1 L) were induced with 0.5 mM IPTG at 37°C for 4 h in a rotary shaker (220 rpm). After centrifugation ($2,739 \times g$, 10 min, 4°C), the cells were resuspended in 300 ml of buffer A (1 \times PBS, 0.2% (v/v) glycerine, 20% (v/v) Triton X-100, 2 mM DTT, pH 7.4) and were sonicated at 400 W for 20 min, until the solution became clear. Following centrifugation at $2,739 \times g$ for 20 min at 4°C , the supernatant was collected. Additionally, the Ni-NTA Sefinose Resin affinity chromatography column was balanced with 10 column volumes of binding buffer B (20 mM Tris-HCl, 8 M urea, 500 mM NaCl, 5 mM imidazole, pH 8.0). The supernatant was loaded into a 10-ml chromatography column with a flow rate of 2 ml/min. Next, the column was washed with 10 column volumes of binding buffer B to remove nonspecific binding hybrid proteins. The target protein was eluted with 500 mM imidazole in binding buffer B using four column volumes for each step. Fractions containing the target protein were identified via SDS-PAGE. At the same time, the protein concentration at each step was determined using the BCA protein assay kit.

Measurement of DPPH Radical Scavenging Activity

The DSAOP antioxidant activity was determined using the DPPH radical scavenging assay according to the methods reported by Memarpour-Yazdi *et al.* [17]. The experimental group (A_i) protein sample (0.2 ml) was added to a sterile 2-ml centrifuge tube, mixed with 0.2 ml of deionized water and 0.4 ml of DPPH solution (0.2 mM in anhydrous ethanol), and vortexed for 10 sec. Next, the tube was incubated for 20 min in the dark at room temperature, and the antioxidant activity was detected at 517 nm. The blank (A_i) and control (A_0) groups were assessed with the same methods under the same conditions. The blank group contained 0.4 ml of anhydrous ethanol in place of the DPPH solution and 0.2 ml of deionized water in place of the protein sample. The control group contained 0.2 ml of deionized water in place of the protein sample. DSAOP samples were serially diluted, added to 96-well plates, and incubated, and the antioxidant activity was detected at 517 nm. Vitamin E was used as a positive control. The DPPH radical scavenging activity was calculated as

follows: DPPH radical scavenging activity (100%) = $[1 - (A_i - A_j)/A_0] \times 100$

Measurement of Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity was measured through improvements in pyrogallol autoxidation [18] with some modifications. Briefly, the sample was mixed with a 25-fold volume of Tris-HCl buffer (pH 8.3) in a clean centrifuge tube. The mixture was incubated in a 25°C water bath for 10 min, an equal volume of pyrogallol was added, and the absorbance was then measured at 325 nm every 30 sec for 5 min. The blank contained an equal volume of deionized water instead of a sample. Vitamin E was used as a positive control. The superoxide radical scavenging activity was calculated as follows: superoxide radical scavenging activity (100%) = $[1 - V_i/V_0] \times 100$. V_i and V_0 represent the slope of the absorbance line for the sample and blank, respectively.

Measurement of Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity was measured according to the methods of Zhuang *et al.* [19] with some modifications. Briefly, the experimental (A_i) DSAOP sample (1.5 ml) was mixed with 0.5 ml of 3 mM ferrous sulfate, and then 0.5 ml of 3 mM salicylic acid and 0.5 ml of 3 mM H_2O_2 solution were added. The mixture was incubated at 37°C for 30 min and the absorbance was measured at 510 nm. The blank (A_i) and control (A_0) groups were measured using the same methods under the same conditions. The blank group contained 0.5 ml of deionized water in place of salicylic acid. The control group contained 1.5 ml of deionized water in place of protein samples. Vitamin E was used as a positive control. The hydroxyl radical scavenging activity was calculated as follows: hydroxyl radical scavenging activity (100%) = $[1 - (A_i - A_j)/A_0] \times 100$.

Pilot-Scale Production of DSAOP

Pilot-scale fermentation experiments were carried out in a 50-L BAILUN automatic system bioreactor according to the methods of Anane *et al.* [20] with some modifications. The cells were precultured in sterile LB liquid medium containing kanamycin. For the DSAOP scale-up preparation, precultured samples were inoculated (5% (v/v)) in fresh LB broth containing kanamycin (50 µg/ml) at 37°C for 4 h. The cells (50 L) were induced with 0.5 mM IPTG at 37°C for 6 h in a rotary shaker (220 rpm). Finally, the corresponding molecular weight of the target recombinant protein was determined using a 12% SDS-PAGE gel to verify successful pilot expression in the 50-L fermentation tank.

Results and Discussion

DSAOP Sequence Biocomputing Analysis

The molecular formula, theoretical molecular mass, and isoelectric point of DSAOP are $C_{481}H_{786}N_{178}O_{156}S_5$, 11,718.93 Da,

and 10.18, respectively. The total number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) are 14 and 20, respectively. The analysis of the secondary structure of the recombinant protein (Fig. 1A) indicated that DSAOP is highly hydrophilic, and the results show the secondary structure of DSAOP has five peaks (score < -0.2) at position sections 20–100. This result means the recombinant protein can be easily stored in powder form using freeze-drying and is easily redissolved when needed. This finding confirms that of Wu *et al.* [11] who prepared antioxidant peptides from *Pinctada fucata* using enzymatic hydrolysis methods, and their peptides had good hydrophilic properties and were easily absorbed. In a previous study, amino acid scales played an important role in the functional activity of antioxidant peptides, which were defined by a numerical value assigned to each type of amino acid [2]. Usually, the most frequently used scales for antioxidant peptide amino acids are the hydrophobicity or hydrophilicity scales, as well as the secondary structure conformational parameter scales, but many other scales exist that are based on different chemical and physical properties of the amino acids. To forecast the

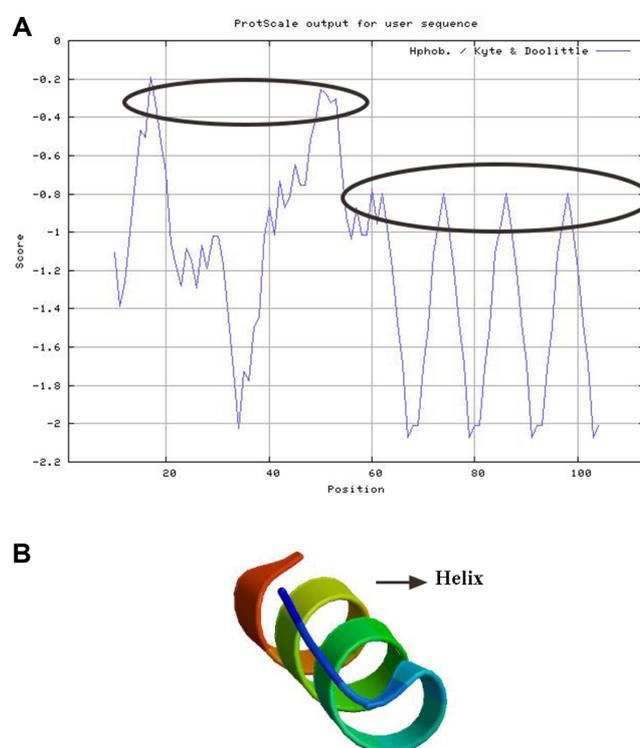


Fig. 1. (A) Structural representation depicting the hydrophobicity of DSAOP secondary structure and (B) 3D structural representation depicting the spatial conformation of DSAOP.

spatial conformation and expression, a 3D structural representation of DSAOP was created with SWISS-MODEL using 3f92.1.A as a template. The results (Fig. 1B) illustrated that DSAOP had spatial conformation with an α -helix after the design, and the results demonstrated that the exposure of the active site amino acids might play an important role in the antioxidant activity. The new spatial conformation containing an α -helix could provide more targeted binding sites for scavenging free radicals and improve the stability of DSAOP containing physical and chemical properties for higher levels of expression and applications. On the other hand, the molecular formula, theoretical molecular mass, and isoelectric point of the AOP were $C_{434}H_{77}N_{17}O_{13}$, 1,040.19 Da, and 10.84, respectively. However, an AOP chain containing only 10 amino acids is too short (at least 30 amino acids) to build a model for predicating the 3D structure. Furthermore, compared with a natural AOP that was isolated and purified from *Pinctada fucata* meat with an enzymatic hydrolysis method, the spatial conformation for DSAOP means that it might improve the antioxidant activity and improve peptide stability against pH, temperature, or light in addition to improving the safety of DSAOP in human cells or skin. A commonly accepted theory is that naturally isolated antioxidant peptides have a substantial advantage in activity and stability compared with chemically synthesized antioxidant peptides. The results of this study are consistent with prior studies conducted by Duan *et al.* [21], who reported that the sequence of the antioxidant peptide HLFGPPGKKDPV derived from eggs had a β -sheet secondary structure that increased its activity and stability.

To summarize, the antioxidant activity of the polypeptide was not only related to its peptide chain length, amino acid composition, amino acid sequence, degree of side chain amino acids glycosylation, size of the side chain structure, and molecular weight, but was also related to spatial conformation. Furthermore, the spatial conformations verified that it might play a more important role during oxidation in human bodies.

Recombinant Plasmid Construction and Expression of DSAOP

To improve the yield, solubility, and expression rate of the target protein in *E. coli*, we fused the N-terminus of DSAOP to a His tag, which has been shown to facilitate purification and causes no interference with the physiological activity of the target protein [22]. To ensure a higher yield, the DSAOP sequence was optimized using high-usage *E. coli* codons. The DSAOP fragment was successfully inserted into the pET30a (+) vector at the BamHI and XhoI restriction

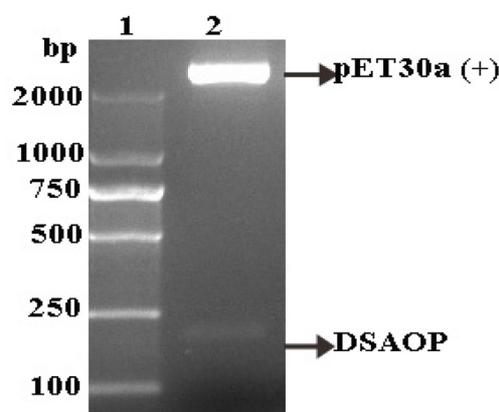


Fig. 2. Agarose gel electrophoresis analysis of recombinant plasmid pET-DSAOP.

Lane 1: DNA Ladder 2000; Lane 2: the double digestion by BamHI and XhoI.

sites with the His tag at the N-terminus of DSAOP, and the recombinant plasmids were verified by double enzyme digestion and DNA sequencing. The results (Fig. 2) showed a nearby 250-bp band DSAOP gene in the 1% agarose gel electrophoresis, which revealed that DSAOP had been successfully ligated into the expression vectors, and the outcome is consistent with the results of Bis *et al.* [23] and Kralicek [24], who expressed human IFN α in *E. coli*. Next, the positively selected recombinant plasmids were transformed into DE3 for protein expression.

For evaluating the expression of recombinant protein DSAOP, IPTG was added to the medium when the engineered bacteria were incubated to a logarithmic phase, and then SDS-PAGE validated whether the target proteins were successfully expressed. The results (Fig. 3A) indicated there was a protein band under 15,000 Da that corresponds to the theoretical molecular mass of the target protein, which was observed only after IPTG induction. The results confirmed successful expression of the recombinant protein DSAOP. This result was corroborated by western blot analysis as well (Fig. 3B). The recombinant protein DSAOP containing the designed gene sequence, linker protein sequence, and His tag (together 12,000 Da) was expressed for this study. The target recombinant protein DSAOP was obtained only in supernatant after induction with IPTG and new bands appeared at the expected molecular weights compared with the control lysate from non-induced lysates. Generally, a higher growth temperature leads to reduced solubility and increased aggregation of target proteins in *E. coli* expression systems. In contrast, a lower growth temperature extends the time required to

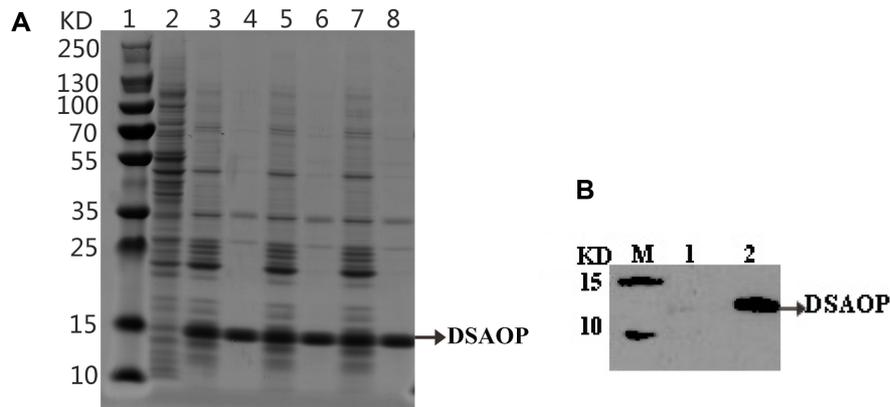


Fig. 3. Expression of the recombinant designed antioxidant peptide from *Pinctada fucata* (DSAOP).

(A) 12% SDS-PAGE analysis of the expression of recombinant DSAOP of 20°C, 30°C, and 37°C. Lane 1: protein molecular weight marker; Lane 2: non-induced lysates; Lane 3: supernatant under 20°C induced for 12 h; Lane 4: supernatant under 20°C induced for 6 h; Lane 5: lysates under 30°C induced for 6 h; Lane 6: supernatant under 30°C induced for 6 h; Lane 7: lysates under 37°C induced for 6 h; Lane 8: supernatant under 37°C induced for 6 h. (B) Western blot analysis of the expression recombinant DSAOP under 37°C induced using an anti-His tag antibody; Lane M: protein molecular weight marker; Lane 1: non-induced supernatant and lysates of DSAOP; Lane 2: induced supernatant of DSAOP.

produce recombinant proteins during fermentation [25]. To evaluate the solubility of the recombinant protein DSAOP, expression was induced at different temperatures using liquid cultures. SDS-PAGE analysis illustrated that DSAOP was soluble at all induction temperatures (Fig. 3A), and there was little difference among the 20°C, 30°C, and 37°C temperatures for inducing soluble DSAOP, which confirmed the biocomputing analysis that indicated DSAOP was hydrophilic and indicates that the target recombinant protein was soluble in this expression system. An induction temperature of 37°C required less time and led to more soluble protein production, and that temperature was chosen to obtain high production of recombinant proteins [26]. Western blot analysis based on the presence of His tags confirmed expression of the recombinant protein by detecting specific protein bands with expected sizes. The results of the western blot assay (Fig. 3B) suggested that the recombinant protein retained its antibody binding activity and reacted with antibodies. Significantly, only antisera from infected mice recognized the recombinant protein on a western blot, whereas no reactivity for the control sera was observed. The recombinant protein DSAOP was localized to the soluble fraction after sonication. Therefore, the peptide could be isolated from the supernatant using the Ni-NTA Sefinose Resin affinity chromatography column.

Purification of Recombinant DSAOP

The high-affinity purification of the His-tagged recombinant

protein was widely used in the pET family in His-tagged plasmid expression vectors as a fusion protein with foreign proteins [27]. For recombinant protein DSAOP purification, precultured samples were inoculated (5% (v/v)) in fresh LB broth containing kanamycin (50 µg/ml) at 37°C for the logarithmic phase. After induction, the cells were resolved, sonicated, and centrifuged to collect the supernatant, including the recombinant protein DSAOP. Then, the supernatant was loaded on a balanced Ni-NTA affinity

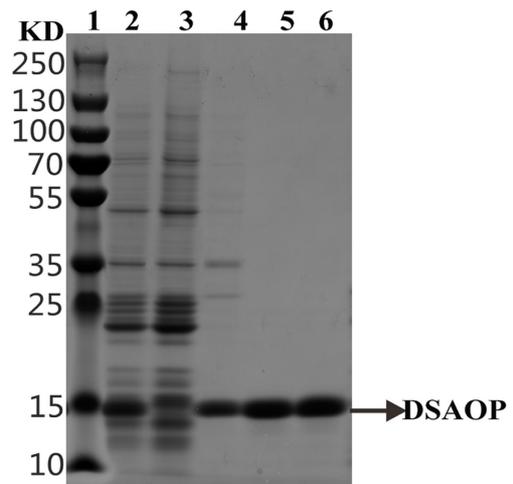


Fig. 4. SDS-PAGE analysis of eluates of the purification process. Lane 1: protein molecular weight marker; Lane 2: supernatant of recombinant protein DSAOP induced at 37°C; Lane 3: column wash-out using binding buffer; Lane 4–6: eluate contained DSAOP.

chromatography column at a flow rate of 2 ml/min, followed by washing and elution steps to collect DSAOP. Fractions containing the recombinant protein were analysed through SDS-PAGE and dialyzed to remove buffer ions. Protein concentration was tested using the BCA protein assay at each step. SDS-PAGE analysis (Fig. 4) illustrated that the recombinant protein DSAOP had few lower molecular weight contaminants and was eluted successfully. The purified DSAOP was obtained after desalination and concentration, and the final purity was more than 95% after using affinity chromatography and this result is consistent with the outcomes of some other studies [28, 29].

Antioxidant Activity Assays

To study the antioxidant activity of the purified recombinant protein DSAOP, the antioxidant activity was investigated through measuring DPPH radical scavenging activity, superoxide radical scavenging activity, and hydroxyl radical scavenging activity. The results (Table 1) showed that the IC₅₀ values for the DPPH radical scavenging activity, superoxide radical scavenging activity, and hydroxyl radical scavenging activity of DSAOP were 0.136 ± 0.006 , 0.625 ± 0.025 , and 0.306 ± 0.015 mg/ml, respectively. Meanwhile, the IC₅₀ of antioxidant activities of natural AOP, chemosynthesized AOP, and vitamin E were also measured as positive controls and negative controls under the same experimental conditions.

The IC₅₀ of DPPH radical scavenging activity results (Table 1) showed that the radical scavenging activities were different for different peptides or vitamin E. Additionally, the recombinant protein DSAOP possessed the same DPPH radical scavenging activity as natural AOP ($p > 0.05$). DSAOP antioxidant activity was lower than that of vitamin E (0.025 ± 0.002 mg/ml), but higher than that of chemosynthesized AOP (0.385 ± 0.008 mg/ml). Meanwhile, vitamin E as an antioxidant in cosmetics has exhibited similar DPPH radical scavenging properties compared with glutathione that is common in cosmetics applications, and this similarity could provide some information to guide DSAOP application in future research studies because these two

antioxidants are both water-soluble and lipid-soluble. Although the DPPH scavenging activity of DSAOP was lower than the activity of vitamin E, which has been used widely as a synthetic antioxidant in cosmetics, it inhibited higher amounts of antioxidant activity that of chemosynthesized AOP, which had a linear structure. This result indicates that the DSAOP sequence with spatial conformations was very important and affected antioxidant activity compared with other elements, such as peptide chain length, amino acid composition, amino acid sequence, the degree of side chain amino acid glycosylation, the size of the side chain structure, and molecular weight. This hypothesis was verified using biocomputing analysis (Fig. 1) that corresponds to other research studies and indicates that natural antioxidant oligopeptides had higher antioxidant activity compared with chemosynthesized oligopeptides [30]. Additionally, the DSAOP obtained by this method exhibited higher DPPH radical scavenging activity compared with AOP prepared by chemical synthesis or enzymatic extraction from marine antioxidant peptides, such as *Gadus morhua* [31], *Theragra chalcogramma* [32], *Catla* [33], salmon [1] and bluefin [34], which had concentrations of 2.5, 2.5, 3.47, 1.63, and 10 mg/ml, respectively.

The superoxide radical scavenging activities of DSAOP, natural AOP, chemosynthesized AOP, and vitamin E were shown to be concentration-dependent (Table 1). Meanwhile, the recombinant protein DSAOP exhibited higher radical scavenging activities than both natural AOP ($p < 0.05$) and chemosynthesized AOP ($p < 0.05$). DSAOP prepared by our methods showed a higher superoxide radical scavenging IC₅₀ value (0.625 ± 0.025 mg/ml) compared with AOP prepared by chemical synthesis (1.175 ± 0.063 mg/ml) or enzymatic extraction of natural AOP (1.046 ± 0.03 mg/ml), but there was a lower superoxide radical scavenging IC₅₀ value for vitamin E (0.305 ± 0.003 mg/ml). In particular, the DSAOP superoxide radical scavenging activity was increased by more than two times compared with chemosynthesized AOP, and the result revealed that the DSAOP natural space structure had a more important role

Table 1. Antioxidant activity of DSAOP, natural AOP, chemosynthesized AOP, and vitamin E.

Antioxidant	DPPH radical scavenging activity (IC ₅₀ , mg/ml)	Superoxide radical scavenging activity (IC ₅₀ , mg/ml)	Hydroxyl radical scavenging activity (IC ₅₀ , mg/ml)
DSAOP	0.136 ± 0.006 b	0.625 ± 0.025 b	0.306 ± 0.015 b
Natural AOP	0.132 ± 0.005 b	1.046 ± 0.03 c	0.268 ± 0.045 a
Chemosynthesized AOP	0.385 ± 0.008 c	1.175 ± 0.063 c	0.975 ± 0.082 c
Vitamin E	0.025 ± 0.002 a	0.305 ± 0.003 a	0.275 ± 0.005 a

Values represent the mean \pm SD ($n = 3$); different letters (a, b) indicate significant differences ($p < 0.05$). IC₅₀: concentration that inhibits enzyme activity by 50%.

for the relationship between structure and function for antioxidant peptides than linear structures. Similarly, the biocomputing analysis (Fig. 1B) showed consistent results corresponding to other research studies, which indicated that natural antioxidant oligopeptides had higher antioxidant activity compared with chemosynthesized oligopeptides [12, 35]. Conversely, DSAOP prepared using the protocol in this study had higher superoxide scavenging activity than some antioxidant peptides from marine sources in other studies. For example, the IC_{50} of superoxide radical scavenging activities of antioxidant peptides from zein [36] and corn [30] were 12.82 and 3.6 mg/ml, respectively.

The hydroxyl radical scavenging abilities of DSAOP, natural AOP, chemosynthesized AOP, and vitamin E were measured under the same experimental conditions. The corresponding IC_{50} values were 0.306 ± 0.015 , 0.268 ± 0.045 , 0.975 ± 0.082 , and 0.275 ± 0.005 mg/ml, respectively (Table 1). The radical scavenging activities were different for antioxidant peptides and vitamin E, and DSAOP showed higher radical scavenging activity than chemosynthesized AOP ($p < 0.05$), as well as lower radical scavenging activity in vitro compared with vitamin E ($p < 0.05$), which indicates that the space structure of DSAOP played a more important role in the relationship between structure and function. This result corresponded to the outcomes of a previous study [15] that used biocomputing analysis (Fig. 1B). Similarly, the hydroxyl scavenging IC_{50} value of DSAOP prepared using recombinant engineering strain techniques had a better spatial conformation compared with chemosynthesis or enzymatic hydrolysis techniques, such as antioxidant peptides from tuna (2 mg/ml) [37], *Oreochromis niloticus* (0.263 mg/ml) [38], horse mackerel (1 mg/ml) [39], and bluefin leatherjacket (1.04 mg/ml) [34].

Stability of the Recombinant DSAOP

To evaluate the antioxidant activity stability of the recombinant protein DSAOP and clarify the relationship between antioxidant activity and structure after design, a DPPH radical scavenging time experiment was undertaken, and vitamin E was used as the positive control. The results (Fig. 5) showed that more free radicals were cleared by DSAOP, natural AOP, chemosynthesized AOP, and vitamin E as scavenging time increased. In particular, the radical scavenging time of natural AOP, chemosynthesized AOP, and vitamin E almost reached a peak in half an hour, then subsequent change was very small. However, the DSAOP radical scavenging value increased until 120 min, and its scavenging time was longer than that of the other three antioxidants, which indicates that the DSAOP space structure

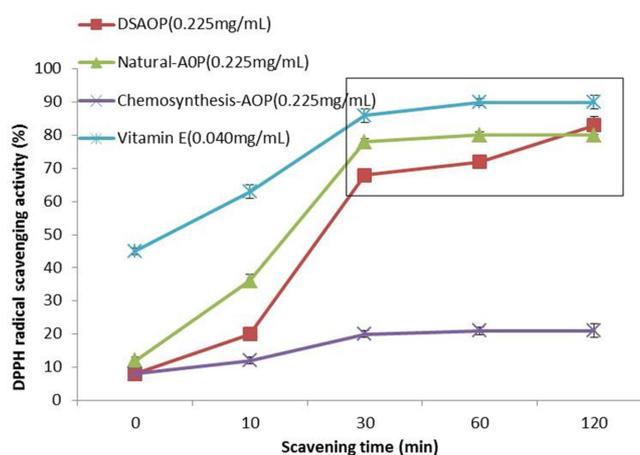


Fig. 5. DPPH radical scavenging activity (100%) of DSAOP^a, natural AOP^a, chemosynthesized AOP^a, and vitamin E^b.

^aScavenging effects were tested at a concentration of 0.225 mg/ml,

^bScavenging effects were tested at a concentration of 0.040 mg/ml.

was stable and had more binding sites for radical-scavenging activity. Sharma and Bhat [40] reported that the radical-scavenging time activity is an important factor when using a DPPH radical scavenging activity assay for evaluating antioxidant activity, and the peptide radical-scavenging time should not be ignored when considering putative applications in cosmetic. Such activity means the recombinant protein DSAOP likely has broad applications in sunscreen cosmetics because of the longer scavenging time, and the peptide could serve as a basis for further characterization and downstream applications of the peptides.

Pilot-Scale Production of Recombinant DSAOP

Although expression of a heterologous protein is conducted in shake-flask culture, protein levels are typically much higher in fermenter cultures. Media containing tryptone, yeast extract, and sodium chloride are economical, and this expression system is nearly ideal for pilot-scale production of heterologous proteins in fermenters [41]. However, the move from a low-density shake flask to high-density, small-volume fermenter cultures using shake-flask culture parameters could achieve ultrahigh production. In the present investigation, the target recombinant protein was obtained in a shake-flask culture, and the optimized fermentation conditions were LB medium (pH 7.0) containing kanamycin (50 mg/ml), a culture temperature of 37°C, an induction temperature of 37°C, a culture time of 4 h, an induction time of 6 h, a shaking speed of 220 rpm, and 0.2 mM IPTG. Therefore, this protocol took parameters of shake-flask culture conditions in this pilot-scale fermentation

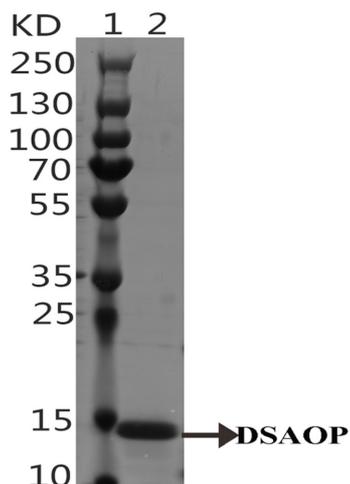


Fig. 6. SDS-PAGE of DSAOP expressed in fermenter culture.

to verify the expression of the produced target protein. In this experiment, a two-stage process is widely used for the production of foreign proteins in fermenter cultures of recombinant *E. coli*. In the first stage, the recombinantly engineered strain DE3-pET-DSAOP was batch-cultured in medium with the optimum growth temperature 37°C to accumulate biomass. The second stage involved a feed-batch transition phase in which IPTG was fed to the culture at the temperature 30°C, which can maintain the natural activity to further increase the biomass concentration until the desired growth is reached and prepare the cells for induction. During the inductions, the strain growth rates, shake speed, and aeration in the fermenter should be monitored closely to maintain high productivity. Finally, a target recombinant protein with a molecular mass corresponding to that of DSAOP was detected using SDS-PAGE, and the results (Fig. 6) were expressed successfully in fermenter cultures. This result is consistent with other research in terms of using this host strain [42], and this protocol could be a basis for pilot scale expression and purification of the recombinant protein for further characterization and downstream applications.

In conclusion, we generated a designed antioxidant peptide from *Pinctada fucata* meat (DSAOP) using recombinant *E. coli* in addition to clarifying the relationship between antioxidant activity and spatial conformation. In contrast to traditional separation and purification methods of enzymatic hydrolysis or chemical synthesis techniques, the DSAOP purification process in this protocol was simple and less time-consuming (12 h), and resulted in higher yields. The spatial conformations played a more important role during oxidation. Furthermore, a scale-up experiment

was carried out and verified that the recombinant protein DSAOP was expressed successfully, which indicated that this prepared method could be easily converted to a wide variety of applications. In addition, for practical applications in nutraceuticals and cosmetics, the stability of DSAOP at different pH values, temperatures, or light conditions as well as the safety of DSAOP in human cells will be further researched in future studies. In summary, the proposed method will increase the availability of DSAOP for research and its potential applications in cosmetic and health food industries. Compared with methods such as chemical synthesis or enzymatic hydrolysis, this method benefited from several advantages in terms of purity, speed of preparation, costs, and degree of automation for preparing antioxidant peptides. Furthermore, this method provides useful information for future characterization and identification of antioxidant peptides from *Pinctada fucata* meat or other marine species.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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