Stability of Ceylon spinach (Basella alba L.) seed protein extract and its effect on the microbiological, chemical and sensory quality of sturgeon fillets stored at 4 °C

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**ABSTRACT**

The antimicrobial activity and preservative effect of Ceylon spinach (Basella alba L.) seed protein extract (CSSPE) had inhibitory effect on 33 microorganisms, including 1 fungus and 32 bacteria (25 gram-negative bacteria and 7 gram-positive bacteria). In addition, CSSPE inhibited bacterial growth (total viable counts, Pseudomonas, Aeromonas and psychrophots), suppressed ATP degradation and retained good-quality characteristic of sturgeon fillets stored at 4°C. The shelf life of 30 mg/mL CSSPE treated fillets was around 7 days according to the results of total viable count, which extended around 2 days compared with that of the control group. The stability of CSSPE was also evaluated, and the results showed that CSSPE had excellent stability under UV irradiation, monovalent metal ions, surfactant and protease treatment. CSSPE could maintain high antimicrobial activity within the range of pH 2–8 and 12–13 and its thermal denaturation temperature was 70°C. The results suggested that CSSPE had the potential to be developed as a preservative for aquatic products.

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**Introduction**

In the last several years, sturgeon (*Acipenseridae*) has been widely farmed because of its fast growth, strong adaptability and high economic value.\(^1\) China accounts for 85% of global sturgeon production.\(^2\) However, sturges are susceptible to spoilage, mainly dominated by the growth of microorganisms.\(^3\) Spoilage of sturgeon has caused hidden dangers of food safety and huge economic losses. Hence, quality deterioration and shortened shelf life of sturgeon are major concerns to consumers and industry. Chemical preservatives have been widely used to inhibit the spoilage process and extend the shelf life of food. However, with the increasing concern for their potential toxicity to humans, the use of chemical preservatives is restricted. Due to the strong demand for food safety, natural preservatives of various origins, including plants, animals, microorganisms and their metabolites, are being developed,\(^4\) and among which, plant resources are regarded as the most promising one.\(^5\)

Proteins, essential oils, alkaloids, flavonoids, polyphenols and polysaccharides extracted from plants have been proved to possess antimicrobial effects.\(^6\)–\(^9\) Antimicrobial proteins or peptides are self-defense products of plants and could eliminate bacteria and fungi by various physiological defensive mechanisms, exhibiting great potential to be used as food preservatives.\(^10\)–\(^13\) Plant seeds contain a range of antimicrobial proteins to protect embryos and seedlings during early development in highly microbial soil environments.\(^5\) Many seed protein extracts from plants, such as *Chenopodium pallidicaule* Aellen and *Benincasa hispida*, have been proved to have antimicrobial activity.\(^14\),\(^15\)

Ceylon spinach (*Basella alba* L.) is a kind of medicinal vegetable with rich active substances.\(^16\) The antimicrobial activity of Ceylon spinach seed proteins and peptides have been revealed in previous studies. For example, two novel antifungal peptides (α- and β-basrubrins) isolated from Ceylon spinach seeds had potent antifungal activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola*, and *Fusarium oxysporum*.\(^17\),\(^18\) However, the inhibitory effect of Ceylon spinach seed proteins or peptides on food spoilage bacteria was unknown, and its potential for application in food preservation need to be investigated. Moreover, qualified food preservatives must adapt to the complex environment of food processing and storage, such as heat, UV irradiation, acidic and alkaline environments, which may reduce the antimicrobial activity of proteins.\(^19\) Hence, the stability of food antimicrobial agent was important. This study aimed to explore the antimicrobial activity of Ceylon spinach seed protein extract (CSSPE) and its preservative effect on sturgeon fillets stored at 4°C. In addition, the stability of the CSSPE under various treatments, including heating, UV irradiation, acidic and alkaline environments, metal ion, surfactant, and protease exposure was also investigated.

**Materials and methods**

**Materials and reagents**

Ceylon spinach seeds were purchased from the Xinfadi seed market, Beijing. Sturgeon (*Acipenseridae*) was purchased from the Qianbaijia market, Beijing. *Escherichia coli* DH5α (*E. coli*), *Pseudomonas aeruginosa*, *Shewanella putrefaciens*, *Aeromonas*, and *Acinetobacter* sp. were obtained from the Beijing Fisheries Research Institute. *Rhodothea glutinis*, *Bacillus subtilis*, *Staphylococcus aureus*, *Lactobacillus*, *Micrococcus luteus*, *Listeria monocytogenes*, *Vibrio parahemolyticus*, *Enterobacter* sp. and other bacteria from rivers (*Comamonas denitrificans*, *Vogesella indigofera*, *Bacillus velezensis*, *Acinetobacter haemolyticus*, *Brevibacillus bourseleensis*, *Acidovorax temperans*, *Kocuria salsicia*, *Diaphorobacter polyhydroyxybutyratevoran*, *Enterococcus hirae*) were provided by the College of Food Science and Technology, Sichuan Agricultural University. Ethylene diamine tetraacetic acid (EDTA) was purchased from Solebo Biotechnology Co., Ltd. (\(\text{NH}_4\)\(_2\)\(\text{SO}_4\), KCl, CaCl\(_2\), NaCl, MgCl\(_2\), FeSO\(_4\) · 7\(\text{H}_2\)O, ZnSO\(_4\) · 7\(\text{H}_2\)O, CuSO\(_4\) · 5\(\text{H}_2\)O, AlCl\(_3\) · 6\(\text{H}_2\)O, LiCl · H\(\text{H}_2\)O, CoCl\(_2\) · 6\(\text{H}_2\)O, NiCl\(_2\) · 6\(\text{H}_2\)O, PbCl\(_2\), MnSO\(_4\) · H\(\text{H}_2\)O, AgNO\(_3\), Tween 20, Tween 80, Span-80, plate count agar (PCA) medium, VRBGA medium, CFC medium, and AEMA medium were purchased from Sinopharm Chemical Reagent Co., LTD. A Bradford protein quantitative kit was purchased from Tiangen Biochemical Technology Co., Ltd.
NaOH and HCl were purchased from Beijing Chemical Plant. Trypsin, pepsin, alkaline protease, papain, adenosine triphosphoric acid (ATP), adenosine diphosphate (ADP), adenylate (AMP), inosinic acid (IMP), hypoxanthine nucleotide (HxR), hypoxanthine (Hx) and their mixed standard were purchased from Sigma (China) Co., Ltd. Neutral protease was purchased from Beijing Aoboxing Biotechnology Co., Ltd.

**CSSPE preparation and determination of protein mass concentration**

The seeds were crushed and soaked in 1.5% EDTA buffer at a ratio of material to liquid of 1:8 for 14 h. After filtration with eight layers of gauze, the solution was centrifuged at 12,000 g for 5 min. The supernatant was collected, and 95% (NH₄)₂SO₄ was added. After 3 h, the sample was centrifuged at 5,000 g for 20 min, and the precipitate was collected. The precipitate was redissolved with deionized water and put into dialysis bag for 48 h in order to remove the (NH₄)₂SO₄. Centrifuged the solution at 5,000 g for 20 min, the supernatant was collected as the CSSPE.

The mass concentration of the protein in solution was determined according to Bradford’s principle of protein quantification.²⁰ (The volume of reagents added are shown in supplement document). The absorbance value was measured with a Multiskan spectrum at 595 nm. The data was recorded, and a standard curve was drawn with protein mass concentration as the abscissa and absorption value as the ordinate. The protein concentration of the sample was calculated from the protein mass concentration standard curve.

**CSSPE antimicrobial analysis**

Thirty-three strains were selected for antimicrobial testing, including fungi (R. glutinis), pathogenic bacteria (L. monocytogenes, M. luteus, S. aureus, V. parahaemolyticus), spoilage organisms (Enterobacter sp., Pseudomonas sp., Aeromonas, S. putrefaciens, B. subtilis and 14 strains of spoilage organisms screened from putrefied fish) and 9 types of bacteria isolated from rivers. V. parahaemolyticus was diluted to 10⁶ CFU/mL with 10 mL of 3% sodium chloride tryptone water, and 1 mL was placed into 10 mL of 3% sodium chloride tryptone agar solid medium for mixing and solidification. Other strains were activated by PCA medium and then diluted to 10⁶ CFU/mL with sterile normal saline, and 1 mL was placed into 10 mL of PCA solid medium for mixing and solidification. The solidified plates were tested with an Oxford cup (outer diameter 8 mm, inner diameter 6 mm, height 10 mm) for antimicrobial analysis. A total of 200 μL of 30 mg/mL CSSPE was added to each cup, and after incubation at 37°C for 18 ~ 24 h, the diameters of the inhibition zones were observed.

**Treatment and storage of sturgeon fillets**

The fish was placed in ice and transported from Qian Baijia market to the laboratory within 0.5 hours. Upon arrival, the whole fish were washed with sterile water after removing the head, guts, skin and cut into fillets with approximate weight of 40 g. The fillets were soaked in CSSPE solution at different concentrations, including 10 mg/mL, 20 mg/mL and 30 mg/mL, for 30 min and denoted C1, C2, C3, respectively, with the control (CK) group soaked in sterile saline at the same time. The fillets were dried with sterile gauze, packed in sterile polyethylene homogeneous bags and stored at 4°C. Samplings were carried out on days 0, 2, 4, 6, 8 and 10, and 3 parallel experiments were conducted for each group.

**Microbiological analysis of sturgeon fillets**

Total viable bacterial counts (TVC) analysis and selective culture of Pseudomonas, Aeromonas and psychrotrophs were carried out according to the method described by Huang et al.²¹ with some modifications. All operations were conducted under sterile conditions. A 5 g sample was homogenized
for 60s in 45 mL of sterile physiological saline (0.85%, W/V). A 10-fold gradient dilution was utilized, and the appropriate dilution was selected for pouring inoculation in the selective medium plate. Three gradients of inoculation were selected for each microorganism. TVC and psychrotrophs were determined using plate count agar (PCA). For TVC, plates were incubated at 30°C for 2 days, while for psychrotrophs, plates were kept at 7°C for 7 days. Pseudomonas were investigated in Pseudomonas CFC selective culture medium after incubation at 20°C for 2 days. Aeromonas were investigated in ampicillin macconey agar base (AMA) at 28°C for 2 days. Colony counting was performed following culture. Microbiological data were transformed to logarithms of the number of colony forming units (log CFU/g).

ATP-related compounds and the K-value of sturgeon fillets

The extraction and HPLC analysis of ATP-related compounds was carried out according to the method proposed by Vázquez-Ortiz et al. with some modifications: a 2 g sample of fish muscle was homogenized with 20 mL of 0.6 M cold perchloric acid solution and centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was collected while the obtained sediment was washed with 10 mL of 0.6 M cold perchloric acid solution and centrifuged under the conditions described above. The operation was repeated once, and the supernatants were combined. The pH value of the solution was set to 6.5–6.8 with 5 M NaOH. The solution was transferred to a 50 mL precooled volumetric flask and diluted with 5% neutral perchloric acid solution (adjusted to pH 6.4 by 25% ammonia) to volume at 4°C. The solution was centrifuged at 8,000 rpm for 10 min at 4°C and filtered through a 0.22 m microporous membrane. The filtrate was collected and stored at −20°C for HPLC analysis.

The extracts of ATP-related compounds were analyzed using an Agilent 1260 liquid chromatograph equipped with an Agilent-C18 column (250 mm×4.6 mm, 5 μm), with the wavelength 254 nm. ATP-related compounds were eluted using 0.05 M phosphate buffer (pH 6.8) at a flow rate of 1.0 mL/min. The injection volume was 20 μL, and elution was performed at 30°C.

Determination of the standard content of ATP degradation correlates: different concentrations (10, 20, 40, 80, 160 and 320 μg/mL) of the standards of ATP degradation correlates, including ATP, ADP, AMP, IMP, HxR and Hx, were determined under the same conditions, and standard maps were drawn. The species and content of various bioamines were determined by comparing the retention time and peak area of samples and standards, respectively. The K value was calculated according to the following formula:

\[ K \text{ value} \% = \left( \frac{(\text{HxR}+\text{Hx})}{(\text{ATP}+\text{ADP}+\text{AMP}+\text{IMP}+\text{HxR}+\text{Hx})} \right) \times 100 \]

Sensory analysis

Sensory analysis of sturgeon fillets with different storage time was performed by a ten-member experienced sensory panel (five females and five males aged from 20–30), according to the method of Warm K et al. with some modifications. Four parameters including texture, odor, color, and tightness of each fillet were evaluated on a scale of 0–3, respectively. The evaluation criteria is shown in Table 1. Score 0 means the fillets were absolutely fresh, and score 12 means the fillets were extremely rotting, with very soft texture, sharply sour and rancid odor, very yellow color and pronounced gapping, that the score of texture, odor, color, and tightness were all 3. Score 6 was the limit of acceptability, if the sensory score over 6, the fillets will not be acceptable.

Effect of pH value, temperature, UV duration, metal ion, surfactant, and protease exposure on the antimicrobial activity of CSSPE

The 30 mg/mL CSSPE solutions were treated with different pH value, temperature, UV duration, metal ions, surfactants and proteases, respectively, followed by measuring the antimicrobial activity with the Oxford Cup method mentioned in section 2.3. Escherichia coli DH5a was used as an indicator
and the untreated protein solution as control. The diameters of the antimicrobial circles were measured after 24 h of culture at 37°C, with 3 repetitions per sample. The relative antimicrobial activity was calculated based on the following formula:

Relative antimicrobial activity/\% = (Diameter of antimicrobial circle in treatment groups (mm)/ Diameter of antimicrobial circle in the control group (mm)) ×100

The specific operation process of CSSPE solution is described as follows: The pH value of the protein solution was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, and 13.0 with 1 mol/L NaOH and HCl and it was placed in a 37°C water bath for 2 h. Then, the pH value of the protein solution was adjusted to 7.0. The protein solution was placed on crushed ice (0°C), in a refrigerator at 4°C and in a water bath heater for 30 min at different temperatures (25, 60, 70, 80, 90 and 100°C), respectively, and cooled to room-temperature (20°C).

The CSSPE solution was irradiated under a 40 W UV lamp (with irradiation distance of 20 cm) for 2, 4, 6, 8, 10, 12 and 24 h. Metal ions such as K⁺, Na⁺, Li⁺, Fe²⁺, Mn²⁺, Ca²⁺ and Al³⁺ were added to the CSSPE solution to achieve a final concentration of metal ions of 10, 20 or 40 mmol/L. The solution was then placed in a 37°C water bath for 2 h. Five kinds of surfactants (EDTA, urea, Tween-20, Tween-80 and Span-80) were added to the CSSPE solution, the final mass fraction of the surfactant was set to 1%, 5% or 10%, and the solution was placed in a 37°C water bath for 2 h. Five kinds of proteases (trypsin, neutral protease, pepsin, alkaline protease, and papain) were added to the solution, the final mass fraction of protease was set to 1, 5 mg/mL or 9 mg/mL, and the solution was placed in a 37°C water bath for 2 h.

**Statistical analysis**

All the experiments were conducted in triplicate. The normality of data was assessed using SPSS Statistics 17.0 and One-way ANOVA. All data were reported as the mean value ± standard deviation of the mean (SD), with $p<0.05$ considered significant. Graphics were generated by GraphPad Prism 7.0 (GraphPad Software Inc. USA).

**Results and discussion**

**Protein mass concentration of the CSSPE**

Bovine serum albumin (BSA) was used as a standard product of protein standard curve. The standard protein assay curve was $y = 0.3135x + 0.3482$ ($R^2 = 0.9913$). In the formula, $x$ is the protein mass concentration, and $y$ is the absorbance value. The concentration of CSSPE was 32.3 mg/mL as calculated by the protein quantitation standard curve.

**Spectrum of antimicrobial activity**

The antimicrobial spectrum of the CSSPE is shown in Table 2. The plates of all test microorganisms had inhibition zone ranged between 14.06 and 39.77 mm. *Bacillus velezensis*, a species of gram-positive bacteria, exhibited the largest inhibition zone diameter (39.77 mm). Other gram-positive bacteria

| Attribute | 0 | 1 | 2 | 3 |
|-----------|---|---|---|---|
| Texture   | Firm and stiff texture. No wateriness | Slightly soft, initial wateriness | Soft, wateriness noticeable | Very soft and pronounced wateriness |
| Odor      | Sharp seaweed and shellfish | Weak seaweed and shellfish | Slightly sour and incipient rancidity | Sharply sour and rancid |
| Color Gapping | Light pink | Grayish | Grey, starting yellow | Very yellow |
|           | No gapping, coherent | Slight gapping but still coherent | Gapping noticeable, disrupted | Gapping pronounced, disrupted |

Table 1. Scheme for evaluating the sensory quality of sturgeon fillets during storage at 4°C.
including *Micrococcus luteus*, *Listeria monocytogenes*, *Bacillus subtilis* and *Kocuria salsicia* had lager inhibition zone with diameters longer than 25 mm. Among gram-negative bacteria, *Shewanella, Enterobacter, Aeromonas and Pseudomonas* were isolated from spoiled sturgeon and had lager inhibition zones with diameters longer than 26 mm, suggesting that CSSPE could inhibit sturgeon spoilage bacteria and had the potential to be used in sturgeon preservation. In addition, the plates of *Rhodotorula glutinis*, a species of fungus, exhibited obviously inhibition zone and the diameter was 24.59 mm. The result indicated that CSSPE inhibited all test microorganisms including 1 fungus and 32 bacteria (25 gram-negative bacteria and 7 gram-positive bacteria). Similarly, the *Basella alba* whole-plant extract was reported to have moderate activity against *Pseudomonas aeruginosa, Bacillus subtilis* while weak response against *Staphylococcus aureus, Micrococcus luteus* and *Escherichia coli*.¹²¹ Suguna et al. confirmed that the aqueous extract of *Basella alba* leaf has a significant activity against bacteria both gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram negative (*Escherichia coli, Klebsiella pneumoniae*). The inhibition zones of 60 mg/mL *B. alba* extract against *S. aureus, B. subtilis, E. coli*, and *K. pneumoniae* were 12 mm, 22 mm, 15 mm and 10 mm, respective.¹²² However, in the current study, the inhibition zones of 30 mg/mL CSSPE against *S. aureus, B. subtilis, and E. coli* were 19.60 mm, 27.32 mm and 17.00 mm, which were larger than that of *Basella alba* leaf extract. It was indicated that the antimicrobial activity of the seed extract was better than that of the leaf extract. Some other plant seeds protein extracts were also reported to have antimicrobial activity. For example, the crude protein extract of *Bauhinia acuminata* L seeds showed strong antimicrobial activity against both gram positive and gram-negative bacteria.¹²³ Pepsin hydrolysis of proteins extracted from *Monodora myristica* seeds were demonstrated to have potent antimicrobial activity.¹²⁴

| Name                             | Classification | Inhibition zone diameter (mm) |
|----------------------------------|----------------|-------------------------------|
| *Rhodotorula glutinis*           | Fungus         | 24.59 ± 2.83                  |
| *Staphylococcus aureus*          | Gram + bacteria| 19.60 ± 0.73                  |
| *Micrococcus luteus*             | Gram + bacteria| 25.98 ± 1.23                  |
| *Listeria monocytogenes*         | Gram + bacteria| 26.27 ± 1.04                  |
| *Vibrio Parahemolyticus*         | Gram – bacteria| 15.24 ± 0.65                  |
| *Bacillus subtilis*              | Gram + bacteria| 27.32 ± 0.57                  |
| *Shewanella putrefaciens*        | Gram – bacteria| 28.01 ± 1.23                  |
| *Enterobacter sp.*               | Gram – bacteria| 26.15 ± 1.07                  |
| *Escherichia coli*               | Gram – bacteria| 17.00 ± 0.55                  |
| *Acinetobacter sp.*              | Gram – bacteria| 20.83 ± 0.99                  |
| *Acinetobacter johnsonii J1*     | Gram – bacteria| 23.84 ± 0.73                  |
| *Acinetobacter johnsonii J3*     | Gram – bacteria| 27.29 ± 1.25                  |
| *Aeromonas sobria*               | Gram – bacteria| 14.18 ± 0.37                  |
| *Aeromonas media S1*             | Gram – bacteria| 16.59 ± 0.58                  |
| *Aeromonas hydrophila L4*        | Gram – bacteria| 19.82 ± 1.47                  |
| *Aeromonas hydrophila X2*        | Gram – bacteria| 15.53 ± 1.07                  |
| *Aeromonas allosaccharophila S2*  | Gram – bacteria| 22.08 ± 1.96                  |
| *Aeromonas allosaccharophila S3*  | Gram – bacteria| 14.06 ± 0.95                  |
| *Aeromonas sp z1*                | Gram – bacteria| 34.45 ± 1.18                  |
| *Aeromonas. veronii P2*          | Gram – bacteria| 16.52 ± 1.08                  |
| *Aeromonas veronii P4*           | Gram – bacteria| 17.12 ± 0.80                  |
| *Aeromonas veronii P7*           | Gram – bacteria| 15.47 ± 0.58                  |
| *Aeromonas veronii P9*           | Gram – bacteria| 17.48 ± 1.35                  |
| *Pseudomonas aeruginosa*         | Gram – bacteria| 30.32 ± 1.40                  |
| *Comamonas denitrificans*        | Gram – bacteria| 28.12 ± 0.55                  |
| *Vogesella indigofera*           | Gram – bacteria| 16.23 ± 0.60                  |
| *Bacillus velezensis*            | Gram + bacteria| 39.77 ± 2.61                  |
| *Acinetobacter haemolyticus*     | Gram – bacteria| 30.26 ± 1.20                  |
| *Brevibacillus borstelensis*     | Gram + bacteria| 17.77 ± 0.87                  |
| *Acidovorax temperans*           | Gram – bacteria| 26.59 ± 0.92                  |
| *Kocuria salsicia*               | Gram + bacteria| 26.37 ± 0.64                  |
| *Diaphorobacter polyhydroxybutyritovans* | Gram – bacteria| 23.74 ± 1.64                  |
| *Enterococcus hirae*             | Gram – bacteria| 27.14 ± 0.42                  |

¹ CSSPE: Ceylon spinach seed protein extract. ² Inhibition zone diameters are denoted by the mean ± S.D.
**Effect of CSSPE on the quality of sturgeon fillets stored at 4°C**

*Microbiological analysis:* The changes in TVC of sturgeon are shown in Figure 1(A). The initial TVC of all samples were less than 3 log CFU/g, indicating that the samples were in good quality. The maximum viable counts for acceptability of fish were 7 log CFU/g during the storage period.[28] On day 2 and day 4, all samples showed the TVC less than 7 log CFU/g. On day 6, the TVC of CK, C1 and C2 groups were 7.70 log CFU/g, 8.17 log CFU/g and 7.21 log CFU/g, respectively, indicating that the fillets have reached shelf life; however, the TVC of C3 group were 6.06 log CFU/g. On day 8, the TVC of C3 group were 7.92 log CFU/g and the fillets reached shelf life. From day 4 to day 8, the TVC of C3 group were significantly lower (\(p<.05\)) than that of the CK, C1 and C2 groups. The results indicated that 30 mg/mL CSSPE had antimicrobial effect and could prolong the shelf life of sturgeon fillets around 2 days.

The changes in *Pseudomonas* count of sturgeon were shown in Figure 1(B). The counts of *Pseudomonas* in all groups showed similar growth trends during storage. The *Pseudomonas* counts of the C3 group were significantly lower (\(p<.05\)) than that of the CK and C1 groups on day 2 and day 4, while the *Pseudomonas* counts of CK, C1 and C2 groups had no significant difference (\(p>.05\)) during the whole storage. Therefore, 30 mg/mL CSSPE tends to be more effective than that of 10 mg/mL and 20 mg/mL in inhibiting *Pseudomonas* growth.

The changes in *Aeromonas* count of sturgeon are shown in Figure 1(C). At day 4, the *Aeromonas* counts of C1, C2, and C3 groups were significantly lower than that of the CK group (\(p<.05\)). On day 8, the C3 group exhibited the lowest *Aeromonas* counts among all groups (\(p<.05\)). The results showed that CSSPE had an inhibitory effect on the growth of *Aeromonas* in sturgeon, and 30 mg/mL CSSPE displayed the best inhibitory effect.

The changes in psychrotrophs count of sturgeon are shown in Figure 1(D). On day 2 and day 4, the psychrotrophs counts of the C2 and C3 groups were similar and both of them were significantly lower than that of the CK and C1 groups (\(p<.05\)). On day 6, all of the CSSPE treatment groups showed

![Figure 1. Changes in TVC (A), *Pseudomonas* count (B), *Aeromonas* count (C) and psychrotrophs count (D) of control and different CSSPE-treated sturgeon fillets during storage at 4°C. (Control: sterile saline-treated sturgeon fillets; CSSPE: Ceylon spinach seed protein extract).](image-url)
significantly lower psychrotrophs counts than that of the CK group \((p<.05)\). The results indicated that CSSPE has antimicrobial effect on psychrotrophs and 20 mg/mL and 30 mg/mL groups exhibited better antimicrobial effect than 10 mg/mL groups.

The growth and metabolism of microorganisms were the major factors that leading to fish deterioration and spoilage.\[3\] In this study, 30 mg/mL CSSPE significantly reduced the counts of *Pseudomonas, Aeromonas* and psychrotrophs of sturgeon fillets compared to the control groups. Similarly, the antimicrobial spectrum analysis of CSSPE described that CSSPE has the satisfactory antimicrobial effect on *Pseudomonas* and *Aeromonas* (subsection 3.2). The spoilage bacteria of chill-stored freshwater fish were generally dominated by psychrotrophic gram-negative bacteria such as *Pseudomonas* and *Aeromonas*, which usually have strong spoilage ability.\[30,31\] Accordingly to the good inhibition effects on spoilage bacteria, CSSPE had the potential to be developed as a new preservative for chill-stored sturgeon or other fish products.

**K-value of sturgeon fillets:** K-value, the ratio of HxR and Hx to the total degradative compounds of ATP, has been widely used to evaluate the freshness of fish.\[32\] Saito et al. suggested that fish products with K-value below 20% were very fresh, those with K-values below 50% were moderately fresh and above 70% were not fresh.\[33\] The changes in the K-values of sturgeon fillets during storage at 4°C are shown in Figure 2. The initial K-values of all samples were approximately 20%. From day 0 to day 6, K-value of all groups showed an obviously increase. The K values of the C3 group were significantly lower \((p<.05)\) than that of CK, C1 and C2 groups during the whole storage. The K values reached to 50% on day 2 for CK and C1 groups, but on day 4 and day 6 for C2 and C3 groups, respectively, indicating that 20 mg/mL and 30 mg/mL CSSPE could inhibit ATP degradation in sturgeon and the inhibitory effect was enhanced with the CSSPE concentration increased. At day 6, the K-values of the CK, C1 and C2 groups reached 80.45%, 76.45% and 70.89%, respectively, and they continuously increased until the end of storage. However, the K-value of the C3 group was only 50.18% at day 6 and was below 70% during the whole storage. It was obviously that 30 mg/mL CSSPE showed the best effect on delaying the increase of K-value and thus maintaining the sturgeon freshness. The bacteria growth and metabolism play an important role in ATP degradative process, for example, Hx formation can be influenced by nucleoside phosphorylase, which was produced by spoilage bacteria of fish.\[34,35\] Consequently, it can be inferred that the lower K-value in C3 groups may be attributed to the lower bacteria counts.

**Sensory evaluation of sturgeon fillets:** The changes in the sensory score of sturgeon fillets during storage are shown in Figure 3. The score of 0 means absolute freshness, and the initial scores of all samples were 0, indicating the sturgeon fillets were fresh. The CK group exhibited significantly higher sensory scores than that of C1, C2 and C3 groups from day 2 to the end storage \((p<.05)\). On day 6 and day 8, C2 and C3 groups displayed significantly lower sensory scores than that of the CK and C1 groups \((p<.05)\). The score of 6 means the maximum consumer acceptability. The sensory scores of CK, C1, C2 and C3 groups reached to 6 at days 6, 7, 8 and 9, respectively, suggesting that 10 mg/mL, 20 mg/mL and 30 mg/mL CSSPE could prolong the shelf life of sturgeon fillets by approximately 1, 2 and 3 days, respectively. The results indicating that CSSPE could retain better quality characteristics of texture, odor, color and tightness of sturgeon, and its preservation effect was increased with the increasing concentration within 10–30 mg/mL, which was according with the results of K value.

In previous study, the Ceylon spinach stems extracts (CSSE) could inhibit *Staphylococcus aureus* and *Escherichia coli*. The shelf life of pork in control and 120 mg/mL CSSE treatment groups stored at 4°C were 4 and 6 days respectively, measured by the total microbial count results. A sensory evaluation showed that fresh pork dipped with CSSE extract was more accepted by consumers. Similar to this study, the 120 mg/mL CSSE extract had antimicrobial properties that can enhance the quality and extend the shelf life of chilled pork for 2 days at 4°C.\[36\] Many other natural extracts had also been reported to have preservation effect. Ranucci et al. reported that the mix of *Punica granatum* and *Citrus spp* extracts could extend the shelf life of pork sausage stored at 4 ± 1°C.\[37\] Vipasha et al. found that the mix of 0.05% rosemary and 0.08% oregano extracts could extend the shelf life of bison strip loin steaks stored at 3°C.\[38\]
Stability of CSSPE

The effect of pH on the stability of CSSPE is shown in Figure 4(A). The relative antimicrobial activity of CSSPE was above 94% in the pH range of 2 to 8 and decreased to less than 82% when the pH increased from 9 to 10 and then recovered to over 93% when the pH increased from 12 to 13. At the specific pH, which is isoelectric point, the surface charge of protein is zero and the protein easily precipitate out.\(^{[39]}\) The isoelectric point depends on the structure and kind of the protein.\(^{[40]}\) In addition, it was reported that most plant antimicrobial peptides are alkaline.\(^{[41]}\) In this study, the relative antimicrobial activity of CSSPE was decreased at pH 9 and 10. It can be speculated that the isoelectric point of some antimicrobial proteins in CSSPE may be approximately 9–10, and the protein precipitation leading to the lower antimicrobial activity of CSSPE.

Figure 2. Changes in the K values of control and different CSSPE-treated sturgeon fillets during storage at 4°C. (Control: sterile saline-treated sturgeon fillets; CSSPE: Ceylon spinach seed protein extract).

Figure 3. Sensory evaluation of control and different CSSPE-treated sturgeon fillets during storage at 4°C. (Control: sterile saline-treated sturgeon fillets; CSSPE: Ceylon spinach seed protein extract. Values are the sum of the scores of each attribute shown in Table 3).

Stability of CSSPE

The effect of pH on the stability of CSSPE is shown in Figure 4(A). The relative antimicrobial activity of CSSPE was above 94% in the pH range of 2 to 8 and decreased to less than 82% when the pH increased from 9 to 10 and then recovered to over 93% when the pH increased from 12 to 13. At the specific pH, which is isoelectric point, the surface charge of protein is zero and the protein easily precipitate out.\(^{[39]}\) The isoelectric point depends on the structure and kind of the protein.\(^{[40]}\) In addition, it was reported that most plant antimicrobial peptides are alkaline.\(^{[41]}\) In this study, the relative antimicrobial activity of CSSPE was decreased at pH 9 and 10. It can be speculated that the isoelectric point of some antimicrobial proteins in CSSPE may be approximately 9–10, and the protein precipitation leading to the lower antimicrobial activity of CSSPE.
Table 3. Effect of metal ions on the antimicrobial activity of CSSPE. 1.

| Metal ion name | 10 mmol/L | 20 mmol/L | 40 mmol/L |
|----------------|-----------|-----------|-----------|
| K+             | 101.61 ± 7.57 | 101.73 ± 3.32 | 107.69 ± 6.52 |
| Na+            | 103.74 ± 8.68 | 99.77 ± 5.05 | 97.35 ± 7.04 |
| Li+            | 103.33 ± 2.22 | 108.45 ± 9.94 | 103.77 ± 2.96 |
| Ag+            | 99.71 ± 9.07 | 100.61 ± 5.28 | 94.11 ± 1.85* |
| Cu2+           | 74.49 ± 2.30a | 95.42 ± 10.61b | 126.94 ± 0.95c |
| Co2+           | 74.94 ± 7.48a | 128.96 ± 3.65b | 156.53 ± 0.06c |
| Ni2+           | 74.67 ± 4.26b | 115.16 ± 6.26b | 144.54 ± 5.42c |
| Ca2+           | 86.47 ± 3.30ab | 79.76 ± 4.85ab | 74.70 ± 1.48a* |
| Mg2+           | 80.92 ± 1.55ab | 77.93 ± 0.99ab | 76.29 ± 2.90a* |
| Fe3+           | 78.04 ± 6.86a | 86.65 ± 4.47a | 106.38 ± 3.77b |
| Zn2+           | 72.94 ± 2.35a | NA         | NA         |
| Pb2+           | 96.83 ± 8.63 | 90.94 ± 0.16a | 85.40 ± 4.11a* |
| Mn2+           | NA         | NA         | NA         |
| Al3+           | 121.22 ± 3.89a | 121.27 ± 12.77a | 127.14 ± 2.98a* |

1: CSSPE: Ceylon spinach seed protein extract. The protein solution was incubated with 10, 20 and 40 mmol/L metal ions at 37°C for 2 hours. Relative antimicrobial activity was measured compared with the untreated protein solution (100 ± 2.63 (%) of relative activity). Relative antimicrobial activity is denoted by the mean ± S.D. *: The symbol indicates a significant difference (p < 0.05) between the experimental and control group. a,b,c: The lower-case letters indicate a significant difference (p < 0.05) among three concentrations.

The effect of temperature on the stability of the CSSPE was shown in Figure 4(B). The relative antimicrobial activity of CSSPE was above 95% with the temperature ranging from 0 to 70°C. Then, the relative antimicrobial activity of CSSPE decreased sharply when the temperature was above 70°C and down to 0 when the temperature reached 90°C. Heating could denature and inactivate proteins and the process was irreversible. It can be speculated that CSSPE started to denature and inactivate when the temperature was above 70°C and was completely inactivated at 90°C. The results indicated that CSSPE could be used in food processing conditions at temperatures below 70°C.

The effect of UV irradiation on the stability of the CSSPE is shown in Figure 4(C). The relative antimicrobial activity of CSSPE was over 96% after UV irradiation from 2 to 24 h, indicating that CSSPE was stable under UV irradiation. The effect of metal ions on the stability of the CSSPE was shown in Table 3. When the CSSPE was treated with monovalent metal ions, including K+, Na+, Li+ and Ag+, its relative antimicrobial activity had no significant difference compared with the control group (p > 0.05). The relative antimicrobial activity of Cu2+, Co2+ and Ni2+ treatment groups were significantly decreased (less than 75%, p < 0.05) in 10 mmol/L and significantly increased (higher than 126%, p < 0.05) in 40 mmol/L. The relative antimicrobial activity of Ca2+ and Mg2+ treatment groups were significantly lower than that of

Figure 4. Effect of pH (A), temperature (B) and UV irradiation (C) on the antimicrobial activity of CSSPE. (Relative antimicrobial activity was measured compared with that of the untreated protein solution 100 ± 3.58 (%) of relative activity); CSSPE: Ceylon spinach seed protein extract.)
the control group (p<.05) at 10, 20, 40 mmol/L. Furthermore, Zn\(^{2+}\) at 20 and 40 mmol/L and Mn\(^{2+}\) at 10, 20, 40 mmol/L could completely deactivate the antimicrobial activity of CSSPE. The relative antimicrobial activity of Al\(^{3+}\) treatment groups was significantly increased (higher than 121%, p<.05) at the concentration of 10, 20 and 40 mmol/L. The results indicated that the CSSPE was stable under monovalent metal ion exposure but sensitive to divalent and trivalent metal ions. Interestingly, 40 mmol/L Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\) and 10, 20 and 40 mmol/L Al\(^{3+}\) could enhance the antimicrobial activity of CSSPE.

The effect of surfactants on the stability of the CSSPE was shown in Table 4. There was no obvious effect of 1%, 5%, 10% Urea; 1%, 10% Tween-20; 10% Tween-80; and 1% Span-80 on the antimicrobial activity of CSSPE compared with the control group. While 1%, 5% and 10% EDTA, 5% Tween-20, 1% and 5% Tween-80, 5% and 10% Span-80 could significantly increase the relative antimicrobial activity of CSSPE (p<.05) compared with the control group. The result showed that EDTA, Tween-20, Tween-80 and Span-80 could significantly improve the antimicrobial activity of CSSPE at some certain concentrations.

The effect of protease on the stability of CSSPE is shown in Table 5. The relative antimicrobial activity of CSSPE treatment with papain, pepsin, trypsin and alkaline protease at concentrations of 1, 5 and 9 mg/mL have no significantly difference with the control group. The relative antimicrobial activity of 9 mg/mL neutral protease treatment group were significantly lower than that of the control group (p<.05), while there were no significantly difference among the 1, and 5 mg/mL neutral protease treatment groups and the control group. The results indicated that CSSPE was stable under 1, 5, and 9 mg/mL papain, pepsin, trypsin and alkaline, and 1 and 5 mg/mL neutral protease treatments.

It was found that *Shiraia bambusicola* extract, which possessed excellent light-induced antimicrobial activity to the gram-positive bacteria, was stable to heat and natural light, but was susceptible under direct sun rays. Metal ions Fe\(^{2+}\), Fe\(^{3+}\), Al\(^{3+}\) and Cu\(^{2+}\) could affect the stability of *Shiraia bambusicola* extract.\(^{[44]}\) Amin et al. reported that the antimicrobial activity of shallot extract was stable at different pH ranging from 4 to 8, but at high alkaline pH (9–11) the stability was reduced. Relative activities of shallot extract at temperature 7 to 121°C were 88 to 100%. The detergents included cetrimide, tween-20, tween-80, sodium dodecyl sulfate, triton x-100, sodium lauryl sulfate, and enzymes included trypsin, pepsin, lipase, amylase had no effect on antimicrobial activity on shallot extract.\(^{[45]}\) In the current study, CSSPE could maintain high antimicrobial activity within the range of pH 2–8 and 12–13 and the temperature range of 0–70°C, in addition, the antimicrobial activity of

| Table 4. Effect of surfactant on antimicrobial activity of CSSPE. |
|---------------------------------------------------------------|
| **Surfactant name** | 1% | 5% | 10% |
| EDTA | 120.28 ± 6.16<sup>a</sup> | 150.35 ± 15.86<sup>b</sup> | 165.65 ± 7.89<sup>b</sup> |
| Urea | 96.07 ± 5.91<sup>ab</sup> | 93.73 ± 2.57<sup>c</sup> | 101.92 ± 2.17<sup>b</sup> |
| Tween-20 | 93.78 ± 4.72<sup>a</sup> | 118.46 ± 1.42<sup>c</sup> | 107.51 ± 4.93<sup>b</sup> |
| Tween-80 | 113.14 ± 1.27<sup>b</sup> | 110.13 ± 3.16<sup>b</sup> | 101.13 ± 1.16<sup>b</sup> |
| Span-80 | 105.19 ± 9.51<sup>a</sup> | 125.65 ± 5.68<sup>b</sup> | 106.50 ± 0.23<sup>a</sup> |

\(^{1}\): CSSPE: Ceylon spinach seed protein extract. The protein solution was incubated with 1%, 5% and 10% surfactants at 37°C for 2 hours. Relative antimicrobial activity was measured compared with the untreated protein solution (100 ± 4.54% (of relative activity). Relative antimicrobial activity is denoted by the mean ± SD. *: The symbol indicates a significant difference (p<.05) between the experimental and control group. a,b,c: The lower-case letters indicate a significant difference (p<.05) among the three concentrations.
CSSPE was not affected by UV irradiation, monovalent metal ions, surfactant and protease. The stability of the above plant extracts was different, which may be related to plant species and extract components.

### Conclusion

CSSPE have broad-spectrum antimicrobial activity, and could maintain the quality of chilled stored sturgeon. 30 mg/mL CSSPE could prolong the shelf life of sturgeon fillets by approximately 2 days. In addition, CSSPE had good heat resistance and was stable under acidic and neutral conditions, UV irradiation, monovalent metal ion exposure, surfactant exposure and protease exposure. Consequently, CSSPE has the potential to be developed as new preservative for sturgeon and other aquatic products.

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### Disclosure statement

The authors declare that they do not have any conflict of interest.

### Data availability

Data available on request from the authors.

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