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Chelation of the Collagen Peptide of Seabass (*Lates calcarifer*) Scales with Calcium and Its Product Development

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Abstract: Seabass (*Lates calcarifer*) is one of the top farmed and raised fish in Taiwan, and fish scales are the main by-product after processing. Fish scales contain high amounts of collagen, which can chelate with minerals and enhance mineral absorption in the human body. Hence, fish scales from seabass were enzymatically hydrolyzed to obtain seabass scale collagen peptide (SBSCP). Calcium, the most consumed mineral supplement, was chelated with SBSCP to form SBSCP-Ca. The optimal conditions for chelation were a peptide/calcium ratio: 1:1 (*w*/*w*), pH 5.0, and 50 °C for 20 min. The conjugated sites were carboxyl and amino groups based on Fourier transform infrared (FTIR) spectroscopy. Scanning electronic microscope/energy-dispersive X-ray spectroscopy (SEM/EDS) evidently showed the alternation of SBSCP's molecular structure after chelation and increased concentrations of metal ions. SBSCP-Ca was stable up to 90 °C and from pH 2.0 to 5.0. The retention rate was 70%, as determined after in vitro digestion. The extracts of blackcurrant or berry-grape seeds were added to neutralize the fishy odor and provide antioxidant ability for commercialization. This is the first complete study of the characteristics of SBSCP-Ca as well as their commercialization.

Keywords: fish scale; collagen peptide; chelation; calcium

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1. Introduction

Around 60–70% of the fish body is not utilized, such as the scales, bones, skin, head, and inner organs, and is treated as waste. However, those by-products contain many highly valued ingredients, such as collagen [1]. Among those by-products, fish scales contain 54.6% protein, and 96% of that protein is collagen [2]. Several studies have successfully purified collagen from fish scales, which included species such as tilapia, milk fish, and sea bass [3–5]. The collagen purified from fish scales also presented the same beneficial functions as those purified from other sources.

The health benefits of collagen have been widely reported [6–8]. Therefore, collagen is commonly used in food, medical, and cosmetic materials, and its annual sale values were around 100 million US dollars in Taiwan, 2018 [9]. The major health benefit claim of collagen is joint health; many reports stated that collagen was able to improve the binding ability of calcium in bones [7], alleviate arthritis [8], and smooth joint movement [6]. Additionally, collagen has been reported to retard skin aging [10] and is commonly used for cosmetic products.

In addition to aforementioned health benefits, fish scale collagen was demonstrated to possess an effective chelating ability of minerals [11–15]. In addition, several research revealed that absorption efficiency of minerals in the human body increased after chelation with peptide or amino acids [16–19]. Among the essential minerals, calcium presents the highest amount in the human body [20]. In addition to being the major ingredient of bones and teeth, calcium is also related to several physiological functions such as muscle contraction, blood clotting, and nerve signal transmitting [21]. Thus, calcium is

a commonly purchased supplement. However, calcium supplement is normally sold in inorganic form and the absorption rate is low since it is easily precipitated in the slightly alkaline environment of the small intestine [22]. Thus, calcium chelated with fish protein has been tested, such as Alaska Pollock [23] and tilapia [24]. The absorption rate of intestine cells for calcium increased 112.7% after chelating with Pollock protein [23]. Similarly, the absorption rate of calcium chelated with the protein hydrolysate of tilapia scales was higher than $CaCl_2$. Furthermore, the absorption rate of $CaCl_2$ decreased to 47.94%, 79.86%, 59.74%, and 75.43% when oxalate, phytate, tannin, and phosphate were added, respectively. However, there was no reduction of absorption rate for the calcium chelated with tilapia scale hydrolysate [24].

Seabass (*Lates calcarifer*) is one of the top farmed raised fish in Taiwan. Its by-products are abundant and even cause environmental issues. Hence, the aims of this study were to utilize the scales of seabass to produce collagen, which is then chelated with calcium to provide the health benefits of collagen itself and enhance the absorption efficiency of calcium. Firstly, the optimal parameters of chelation, such as mass ratio of collagen/enzyme, pH, temperature, and time were determined. Furthermore, the chelation was investigated by Fourier transform infrared spectra (FT-IR) and scanning electron microscope/energy-dispersive X-ray spectroscopy (SEM/EDS). Plant extracts such as berry-grape seeds were added to improve taste and offer antioxidative capacity to be commercialized.

2. Material and Methods

2.1. Preparation of Fish Scales Collagen

The fish scales of seabass were provided by Mammafisch Co. (Kaohsiung, Taiwan) and the protease mixture was composed of protease N, protease A, protame, and papain. The ratio of enzymes, fish scales, and water was 1:40:240 ($w/w/w$). After incubation for 90 min at pH 7.0 and 50 °C, the mixture was heated at 100 °C for 10 min to inactivate protease. The hydrolysate was filtered through cheesecloth and filter paper (No. 1, Advantec, Tokyo, Japan). The metal ions in the hydrolysate were removed by going through 2% Chelex-100 resin and peptide concentrations were determined by the OPA method.

2.2. Analysis of Molecular Weight

The molecular weights of collagen mixture were determined using modified methods of Wu et al. [5]. The size-exclusion column (Biosep-SEC-S2000, Phenomenex, Co., Torrance, CA, USA) and acetonitrile (45%, v/v) was used for the separation and mobile phase liquid, respectively. The speed of the mobile phase liquid was 1.0 mL/min, retention time was 40 min. The molecular weight of collagen was obtained after comparison with standards at 13,700 Da, 6500 Da, 760 Da, 612.63 Da, and 307 Da.

2.3. Determined the Optimal Parameters of Chelation for Seabass Scale Collagen Peptide (SBSCP) and Calcium

The chelation procedures were based on Hsieh [25]. Firstly, different mass ratios at 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, and 3:1 of SBSCP and $CaCl_2$ (5 mM) were tested. The same volume of phosphate buffer (0.2 M) was added into the mixture of SBSCP and $CaCl_2$, then reacted at pH4.0 and 50 °C in a shaking water bath (130 RPM) for 30 min. Secondly, different pH values, 2, 3, 4, 5, 6, and 7, were tested at 50 °C for 30 min with the mass ratio obtained from the first test. The following tests included different reaction times: 20, 30, 40, 50, and 60 min, as well as different temperatures: 30, 40, 50, 60, and 70 °C with the optimal parameters obtained from the previous tests.

The calcium concentrations in the chelated SBSCP were determined by the o-cresolphthalein complexone (o-CPC) method [25]. After the chelated products were centrifuged at 14,000× g and 4 °C for 15 min, the supernatant was precipitated again by adding equal amounts of ethanol (99%) and centrifugation (14,000× g, 4 °C, 15 min). The calcium amounts of the first and second precipitates as well as the supernatant were measured. The chelating rate was determined as the calcium amount of the second precipitate divided by the total

calcium amount of the two precipitates and supernatant. The chelated product of SBSCP with calcium (SBSCP-Ca) was lyophilized and stored at 4 °C for the following tests.

2.4. Evaluate the Spectrum of Fourier Transform-Infrared (FTIR)

The lyophilized SBSCP-Ca was mixed and ground with KBr powder (1:50; *w*/*w*) into a fine powder under dry conditions, then pressed to form thin and transparent flakes. The frequency of FTIR (FT-730, Horiba, Tokyo, Japan) was set from 400 to 4000 cm^{-1} and tests were conducted in a temperature-controlled room ranging 25–28 °C.

2.5. Observation under the Scanning Electron Microscope (SEM)/Energy Dispersive Spectrometer (EDS)

After being spread on a glass slide by double-side adhesive tape and coated with gold, the samples were observed under a SEM (S3000N, Hitachi, Tokyo, Japan) to study the microstructure of SBSCP and SBSCP-Ca. The elemental compositions of SBSCP and SBSCP-Ca were studied with an EDS (Emax Energy, Hiroba Ltd., Kyoto, Japan).

2.6. The Stability of SBSCP-Ca

The lyophilized SBSCP-Ca was dissolved in ultrapure water at 5 mg/mL for the stability tests, which included pH, temperatures, and in vitro digestion. The procedures were based on Wu et al. [13].

2.6.1. The stability of SBSCP-Ca in Different pH Values

The pH values of the SBSCP-Ca solution were adjusted to 2, 3, 4, 5, 6, or 7, then placed in a water bath at 37 °C for 1 h. After adding ethanol and centrifuged as previously described, the calcium contents were determined. The pH-unadjusted SBSCP-Ca solution was used as a control.

2.6.2. The Stability of SBSCP-Ca in Different Temperatures

The pH-unadjusted SBSCP-Ca solution was placed in a water bath at 50, 60, 70, 80, or 90 °C for 1 h, then the calcium contents were determined as previously described.

2.6.3. In Vitro Simulation of Gastrointestinal Digestion of SBSCP-Ca

The pH of SBSCP-Ca solution was adjusted to 2.0 and 7.0, then pepsin (1%, *w*/*w*) and trypsin were added for gastric and intestinal stability, respectively. The mixture was incubated at 37 °C for 0, 30, 60, 90, or 120 min, then 100 °C for 10 min to inactivate pepsin or trypsin. Calcium content was determined by the procedures previously described. The stability was calculated from the amount of calcium before and after the test.

2.7. Sensory Evaluation and Antioxidative Capacity of the Mixture of SBSCP-Ca and Plant Extracts

2.7.1. Sensory Evaluation

Three plant extracts, blackcurrant, berry-grape seed, and calendula were mixed with SBSCP-Ca to neutralize fishy odor and enhance antioxidative capacity. Different ratios of SBSCP-Ca and plant extract, 2:8, 3:7, 4:6, and 5:5 (*v*/*v*), were evaluated to determine the optimal ratio. The samples were served at room temperature and the color, aroma, flavor, fishy odor, and overall acceptance were evaluated. Before sensory evaluation, 15 panelists were trained by tasting the original SBSCP-Ca solution and then the mixture with plant extracts. The scores ranged from 1 to 9 (1 = extremely dislike, 9 = extremely like). A higher score indicated a higher fishy odor.

2.7.2. Analyses of Antioxidative Capacity

The antioxidative capacity of the mixture of collagen and SBSCP-Ca was measured by DPPH and ABTS radical scavenging tests based on Chou et al. [3]. For the DPPH test, 100 μL of the mixture was added into 400 μL ethanol (95%) and 500 μL DPPH (0.25 mM). After

incubation in the dark for 20 min, absorbance at 517 nm (A_{517}) was measured (Genesys10S, Thermo-Fisher, Waltham, MA, USA). One hundred microliters of water and ascorbic acid (1 mg/mL) were used for the blank and control, respectively. The antioxidant capacity was calculated with the following equation: scavenging capacity = [1 − (A_{517} of sample/A_{517} of the control)] × 100%. For the ABTS test, 10 µL of the mixture was mixed with 990 µL of ABTS solution (2.0 mM). After incubating in the dark for 10 min, absorbance at 737 nm (A_{737}) was measured. Water and Trolox (8 mg/mL) were used as the blank and control, respectively. The scavenging effect was calculated with the following formula: scavenging capacity = [1 − (A_{737} of sample/A_{737} of the control)] × 100%. Triplicate samples were used for each flavor for each test.

2.7.3. Granulation of the Mixture of SBSCP-Ca and Plant Extracts

The mixture, SBSCP-Ca, and plant extracts were blended with maltodextrin (1:3, *v*/*w*), then processed in a dry granulation device (YS-FDG-2, INORA, Taichung, Taiwan) at 70 °C.

2.8. Statistical Analyses

The average and standard deviation were obtained and analyzed by IBM SPSS program (version 22.0, St. Armonk, NY, USA). The significant differences between treatments (p = 0.05.) was conducted by one-way ANOVA and Duncan's test. Triplicate samples were used for each treatment and all experiments were conducted at least twice.

3. Results and Discussion

3.1. Molecular Weight of SBSCP

The molecular weight of SBSCP ranged from 307 to 760 Da, which decreased greatly from the molecular weight (10 kDa) before hydrolyzation (S1). The decrease of molecular weight after hydrolyzation and the range of molecular weight were similar with our previous study, which also used seabass scales as the collagen source [3] and other studies using mullet, milk fish, and tilapia [4,5].

3.2. Chelating Parameters of SBSCP and Calcium

The chelating rate of SBSCP and calcium increased significantly from 82.94 to 92.98% ($p < 0.05$) when mass ratio was adjusted from 1:5 to 1:1 (*w*/*w*). However, when mass ratio of SBSCP and calcium increased to 2:1, the difference in chelating rates were not significant ($p > 0.05$). Thus, a 1:1 mass ratio was determined as the optimal ratio (Figure 1a). Huang et al. [26] also reported that the chelating rates increased from 16.31% to 61.06% when the mass ratio of egg albumen and calcium were raised from 1:1 to 2:1. However, the chelating rates did not change significantly by increasing the mass ratio to 3:1. Similarly, chelating rates increased significantly when the mass ratio of the hydrolysate of sea cucumber egg and calcium was elevated from 1:1 to 3:1 but no significant change was observed by raising the mass ratio to 5:1 [27].

The chelating rates were not significantly different in the pH range from 2.0 to 5.0 and the highest rate was at 5.0. In contrast, the rates decreased greatly at pH 6.0 and 7.0 (Figure 1b). Hsieh [25] also showed that the highest chelating rate for SBSCP and calcium was at pH 4.0 and decreased at neutral and slight alkali pH values. For reacting time periods (Figure 1c) and temperatures (Figure 1d), no significant difference was observed. Other studies also demonstrated insignificant effects from the same range of time periods [28] and temperatures [29].

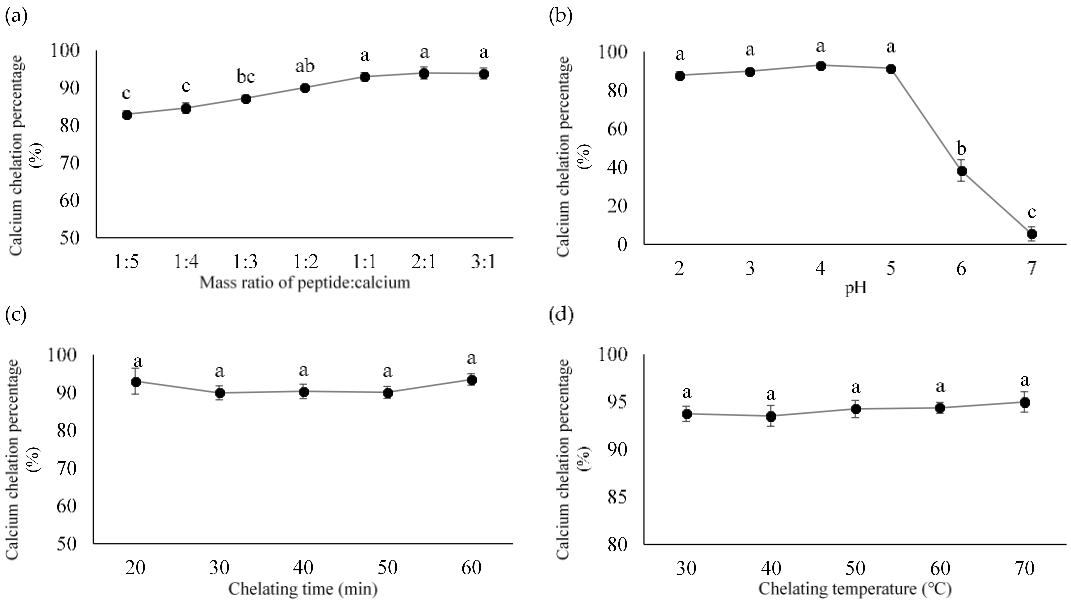

Figure 1. SBSCP-Ca chelation conditions. (**a**) mass ratio (pH 4.0, 50 °C, 30 min), (**b**) pH (1:1, 50 °C, 30 min), (**c**) chelation time (1:1, pH 5.0, 50 °C), (**d**) Chelation temperature (1:1, pH 5.0, 20 min). Different letters indicate significant difference ($p < 0.05$) between treatments.

3.3. FT-IR Analyses

FTIR has been used to observe the absorbance change in the range of frequency from 400 to 4000 cm^{-1}, which results could reveal the structure changes of functional groups, such as O–H, N–H, and C=O, the potential conjugated sites for calcium ions [30]. In this study, the results of FT-IR showed several alterations for the absorption peaks of SBSCP and SBSCP-Ca (Figure 2) and signaled the conjugated sites between peptide and calcium. The absorption peaks shifted at 3332.39 to 3384.46 cm^{-1} indicating the conjugation of NH_2 and Ca^{2+}, in which N–H was replaced by N–Ca as suggested by Lin et al. [31]. Additionally, the shifts at 1658.48 to 1652.70 cm^{-1} indicated the involvement of the C=O bond in carboxyl groups. Furthermore, the shifts at 1542.77 to 1540.85 cm^{-1} suggested the stretching vibration of the N-H bond in amine groups and suggested the involvement of chelation, such as described by Fang et al. [32]. Based on these findings, the chelation mainly occurred in the carboxyl and amine groups of the peptides, which were also presented by previous studies [14,33–35].

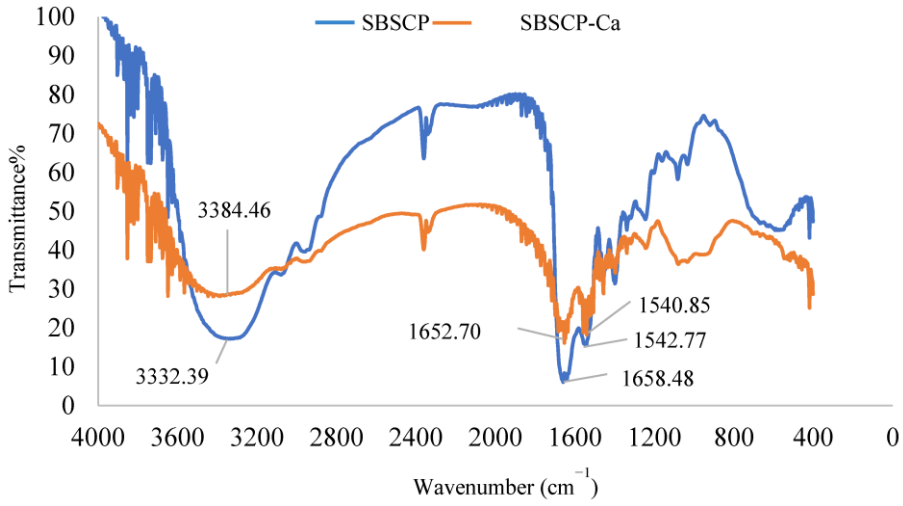

Figure 2. The Fourier transform infrared spectra (FTIR) of SBSCP-Ca.

3.4. Scanning Electron Microscopy and Energy Dispersive Spectrometer (EDS)

The SEM images and EDS analyses revealed the alteration of SBSCP microstructure and element composition before and after chelating with calcium (Figure 3). SBSCP exhibited a flaky structure and a smooth surface. In contrast, SBSCP-Ca demonstrated a granular structure with many branch-like aggregates. Similar results were reported in previous studies [13,36], which proposed that the conjugations of metal ions and the functional groups of peptides altered the intramolecular forces and the surface tension. Additionally, the intermolecular forces, such as the hydrogen bonds of the functional groups that were surrounded with water molecules and nearby peptides, were interrupted, and caused the peptide to aggregate. EDS analyses also demonstrated that the percentages of calcium increased from non-detectable to 3.16% after chelation, thus confirming the conjugation of calcium ions. The calcium percentage corresponded with an earlier study [37], in which the calcium content was at 3.86% after the chelation of collagen peptides of bovine bones with calcium.

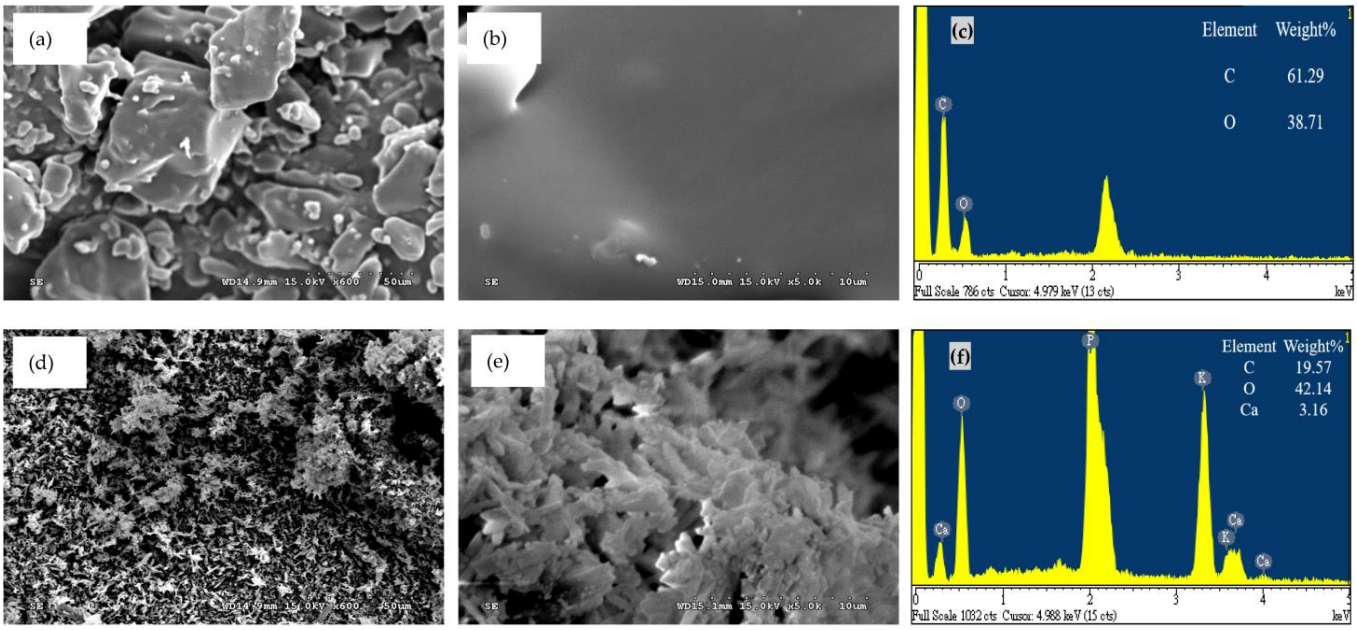

Figure 3. SEM and EDS analysis. (**a**) SEM photograph of SBSCP 600X; (**b**) SEM photograph of SBSCP 5000X; (**c**) EDS image of SBSCP; (**d**) SEM photograph of SBSCP-Ca 600X; (**e**) SEM photograph of SBSCP-Ca 5000X; and (**f**) EDS image of SBSCP-Ca.

3.5. Stability of SBSCP-Ca during Storage

No significant loss ($p > 0.05$) for calcium retention until pH was above 6.0 and temperature reached 90 °C (Figure 4a,b). Similar results were shown for the zinc-chelated krill collagen that was stable in acidic condition (pH 2.0–4.0) but unstable when pH raised to 8.0 [38]. It could be the result of the precipitating reaction of OH^- ions in an alkaline solution and two divalent ions, such as Zn^{2+} and Ca^{2+} [38,39]. The wide range of temperature stability for Ca-chelated collagen was also presented in previous research [28,29,39].

Calcium retention reduced significantly ($p < 0.05$) in the simulated gastric and intestinal digestion, particularly in the intestinal digestion whose condition is alkaline (Figure 4c,d). Calcium chelation was stable in the simulated gastric solution and the retention rate of calcium was 83.90% and 80.81%, after 30 and 120 min, respectively. However, calcium retention reduced rapidly to 76.00% after 30 min the simulated intestinal solution and maintained steadily to 72.59% after 120 min. The rapid reduction of calcium retention could be due to the alkaline condition in the simulated intestinal solution.

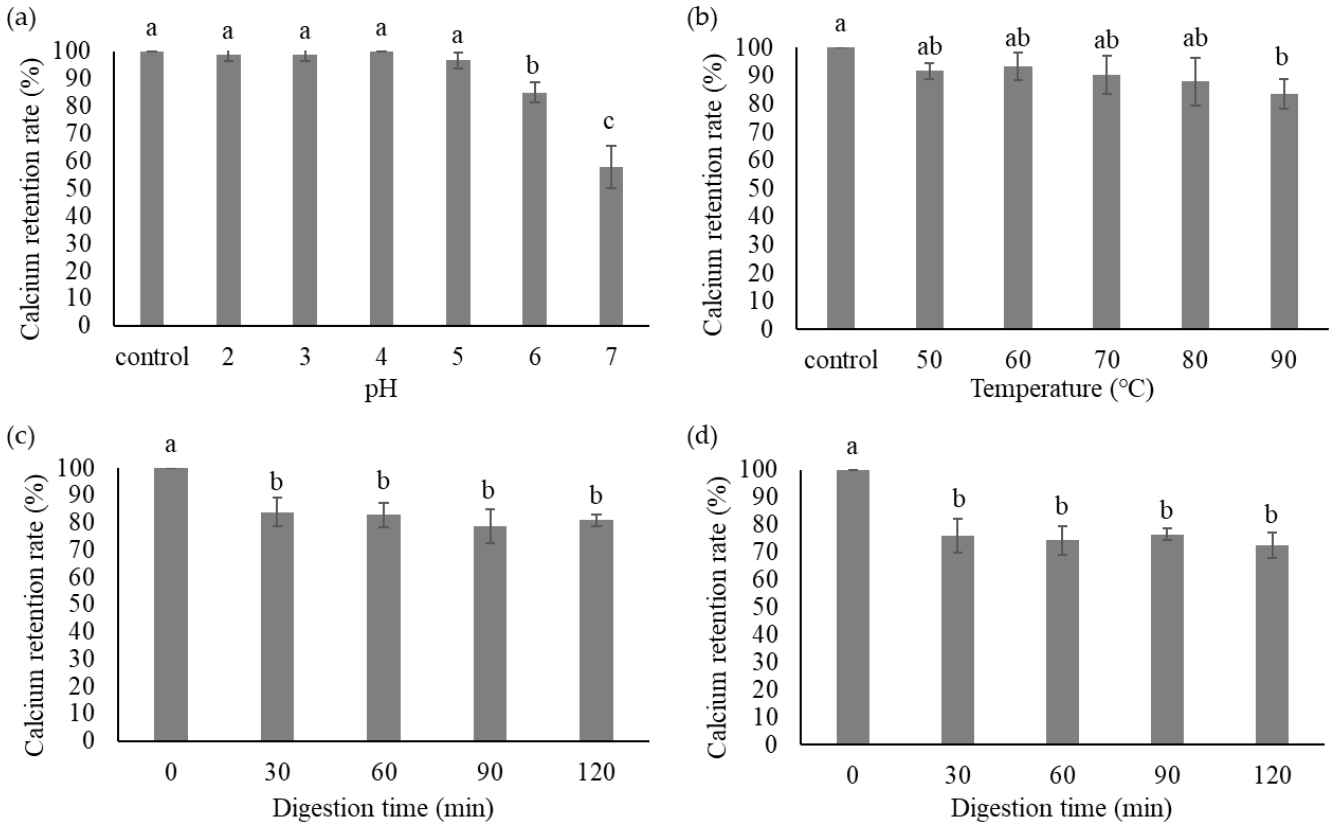

Figure 4. Stability of SBSCP-Ca at various (**a**) pH, (**b**) temperature and in vitro digestion of simulated gastric (**c**) and intestinal (**d**) condition. Different letters indicate significant difference ($p < 0.05$) between treatments.

These results contrasted with a previous study [40], in which cattle bone collagen chelated with calcium showed much higher stability in the simulated intestinal digestion than the gastric one. Zhang et al. [40] proposed that calcium was released more easily in the acidic condition of the gastric environment than in the slightly alkaline intestinal condition since the chelated bone collagen also demonstrated higher stability in alkaline conditions. However, SBSCP-Ca showed higher stability in acidic conditions in this study. Its difference could be due to the different nature of cattle bone collagen and fish scale collagen because other studies showed the chelated products by using collagen from fishes [28,31,39] had higher stability in acidic condition. As for continuously digestion stability, those studies demonstrated around 76–80% and 70% retention rates for calcium [28,39] and ferrous [31], respectively. Our study showed the individual stability of the chelated product of fish collagen-calcium to understand which section of digestion tract affecting the stability of chelated products more. Based on the results, the fish collagen chelated products were more stable in gastric condition than intestinal condition.

3.6. Sensory Evaluation and Stability of the Mixture of Plant-Extracts and SBSCP-Ca Granules during Storage

Among the three plant extracts, blackcurrant, berry-grape seed, and calendula, calendula scored the lowest points for all categories of sensory evaluation. These results could result from the weedy flavor of calendula. Additionally, the mixtures of blackcurrant and berry-grape seed possess sweeter flavor and stronger aroma. Both showed high scores and effectively masked the fish odor (Table 1). Thus, only the mixtures of blackcurrant and berry-grape seed were granulated. The mixture of blackcurrant and SBSCP-Ca showed 37.91% and 58.22% of DPPH and ABTS scavenging capacities of the control, ascorbic acid. Similarly, the mixture of berry-grape seed and SBSCP-Ca showed 68.77% and 54.74% of DPPH and ABTS scavenging capacities of the control. In contrast, the SBSCP-Ca without

adding plant extracts show none and 8.72% of DPPH and ABTS scavenging capacities (Table 2). These were due to blackcurrant and berry-grape seed both containing abundant antioxidative substances, such as anthocyanin, polyphenols, and flavanols [41].

Table 1. Sensory evaluation of SBSCP-Ca products with different plant extracts.

Plant extracts	Color	Aroma	Taste	Fish Odor	Overall Acceptance
Blackcurrant	7.6 ± 1.13^{a}	8.1 ± 0.38^{a}	8.4 ± 0.79^{a}	1.7 ± 1.25^{a}	8.3 ± 0.76^{a}
Berry-grape seed	8.0 ± 0.82^{a}	7.9 ± 1.46^{a}	7.7 ± 0.95^{a}	1.3 ± 0.76^{a}	8.1 ± 0.90^{a}
Calendula	7.1 ± 1.21^{a}	6.3 ± 1.50^{b}	4.9 ± 1.95^{b}	3.3 ± 1.89^{a}	4.9 ± 1.86^{b}

Values represent means and standard deviations of twelve replications (n = 15). Means with the different small case letters in the same column are significantly different ($p < 0.05$). The scale of sensory evaluation is 1 to 9; 1 = extremely dislike, 9 = extremely like.

Table 2. Antioxidant efficacy of SBSCP-Ca plant extract complexes (%).

Peptide Chelation	Plant Extract	DPPH Scavenging (%)	ABTS Scavenging (%)
SBSCP-Ca	Control	89.06 ± 0.003	94.09 ± 0.001
	Non	ND	8.72 ± 0.046^{b}
	Blackcurrant	37.91 ± 0.018^{b}	58.22 ± 0.064^{a}
	Berry-grape seed	68.77 ± 0.016^{a}	54.74 ± 0.055^{a}

DPPH control is Vitamin C (0.125 mg/mL), The ABTS control is Trolox (2 mg/mL). Non refers to SBSCP-Ca without adding plant extract. Values represent means and standard deviations of four replications (n = 4). Means with the different small case letters in the same row are significantly different ($p < 0.05$).

The granular products of SBSCP-Ca after adding plant extracts were stable during storage at 25 or 50 °C for 180 days. Their bacterial population, water content, water activity, and sensory characteristics were not significantly different ($p > 0.05$) throughout the storage (S2).

As aforementioned, collagen itself possesses several healthy functions, particularly for joint and skin care. In addition, chelating with calcium elevates the absorption rate of calcium. This study successfully obtained a collagen-calcium product, which showed high sensory scores and demonstrated excellent stability during digestion test and storage. Thus, this chelated product possesses high potential value for the market of functional food. Furthermore, collagen is obtained from fish scales, a by-product of fish processing. It fits the principle of a sustainable economy, which emphasizes on reutilizing by-product and reducing waste.

The results clearly showed that the products possessed commercial potential. However, the fishy odor is the main obstacle for consumer acceptance. Plant extracts are suitable substances to neutralize the fishy odor but only the ones with strong flavors are appropriate. In this study, three plant extracts were tested but only the extracts of blackcurrant and berry-grape seed were suitable. Though calendula is rich in lutein and popular in the functional food market, its weak flavor was unable to mask the fishy odor effectively. Coupling with granulation, the percentage of collagen reduces. Thus, other types of products, such as a jelly-like, processing method or spray drying, should be considered for commercialization.

4. Conclusions

Collagen peptides with molecular weight ranging from 307 to 760 Da were obtained from seabass fish scales. Calcium, the most consumed mineral supplement, was chelated onto seabass scale collagen peptide (SBSCP). The optimal parameters of chelation were determined. The chelating sites confirmed by FTIR were the carboxyl and amino groups. Additionally, SEM observation showed that SBSCP surface was altered from smooth to a rough and granular appearance with many branch-like aggregates after chelation. EDS also revealed the change of atom composition after chelation. Those results all confirmed the conjugation between calcium and SBSCP. The chelated molecules of SBSCP-Ca were

stable in acidic environments and up to 90 °C. In addition, SBSCP-Ca was highly stable in the simulated gastric solution but less stable in the intestinal solution. The plant extracts of blackcurrant or berry-grape seeds effectively enhanced the sensory characteristics and provided antioxidative abilities. The granular products of the plant extracts and SBSCP-Ca were stable and suitable for commercialization. This study offered the first complete results of SBSCP-Ca characteristics and offered a product with commercial potential.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15086653/s1.

Author Contributions: Conceptualization, C.-M.L. and J.-M.K.; methodology, C.-M.L., J.-M.K., X.-H.Y., S.-G.C. and Y.-T.C.; software, X.-H.Y., Y.-T.C. and S.-G.C.; validation C.-M.L. and J.-M.K.; formal analysis, X.-H.Y., Y.-T.C. and C.-M.L.; investigation, X.-H.Y., Y.-T.C. and C.-M.L.; resources, C.-M.L.; data curation, X.-H.Y., Y.-T.C., S.-G.C. and C.-M.L.; writing—original draft preparation, X.-H.Y.; writing—review and editing, C.-M.L.; visualization, C.-M.L.; supervision, C.-M.L.; project administration, C.-M.L.; funding acquisition, C.-M.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to restriction of funding source.

Conflicts of Interest: The authors declare no conflict of interest.

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