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Extraction of Proteins with Low Fluoride Level from Antarctic Krill (*Euphausia superba*) and Their Composition Analysis

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ABSTRACT: The extraction of proteins with low fluoride level (LFP) from Antarctic krill (*Euphausia superba*) was investigated in this work. The optimal conditions for protein solubilization were determined to be pH 11.5 and 4 °C. The proteins were solubilized two times; a water/krill ratio (mL/g) of 6 and a time of 30 min were used for the first step, whereas the second used a water/krill residue ratio (mL/g) of 3 and a time of 30 min. The optimum pH for protein precipitation was 4.6. A LFP with fluoride content of 9.86 mg/kg (dry weight) was finally obtained through a fluoride removal program. The protein yield of LFP was 52.68%. Composition analysis of LFP indicated it was composed of 66.96% of crude proteins (dry weight) and 33.01% of total lipids (dry weight)„ and all nine essential amino acids were in sufficient amounts to meet FAO/WHO/UNU requirements for adults and infants. In addition, LFP could be taken as a good source of EPA and DHA for consideration of use as a food item for human consumption.

KEYWORDS: *Euphausia superba*, krill proteins, fluoride, protein solubilization, protein precipitation, fluoride removal

INTRODUCTION

Antarctic krill (*Euphausia superba*) is the largest animal protein resource in the world. The total biomass and annual gross postlarval production of Antarctic krill are 117−379 and 342−536 million metric tons, respectively.[1] According to a report of the Food and Agriculture Organization of the United Nations, the total annual capture of all fisheries including wild fisheries and aquaculture has been around 130 million metric tons since 2000.[2] As conventional wild fisheries from nearshore areas have declined due to overfishing in recent years and as aquaculture continues to grow, there will be a greater emphasis on harvesting species such as krill from remote Antarctic ocean area. Antarctic krill contains 77.9−83.1% moisture, 11.9−15.4% proteins, 0.4−3.6% lipids, and about 2% chitin.[3] Antarctic krill contains all of the essential amino acids, and the total essential amino acids for Antarctic krill is 212.1 mg/g protein.[4] The biological value (BV) of krill proteins has been reported to be higher than the BVs of other meat proteins and milk proteins (e.g., casein), but lower than egg proteins.[5]

Although Antarctic krill has a large biomass and high-quality proteins, only about 12% of the total krill is consumed by mankind.[6] Currently, most of the krill in all of the commercial fisheries is used for the manufacture of aquaculture feeds due to its high asthaxantin content and limited potential as food for humans.[7,8] Meat from krill is typically recovered by mechanical deshelling. However, the yield is extremely low, ranging between 10 and 15% on the basis of whole krill weight.[9] Because krill contains strong proteolytic enzymes[10] and a relatively high concentration of water-soluble proteins,[5] krill surimi has not been commercialized. Krill protein concentrates with a protein yield of 45−50% are recovered from whole Antarctic krill by alkali solubilization and acid precipitation. This yield is higher than surimi processing.[4] The recovered krill proteins are a high-quality protein that is suitable for foods designated for adult and infant consumption.[11] However, a high fluoride content was found in Antarctic krill. Fluoride concentration was 1102−1432 μg/g (dry weight) in whole krill, 3828−4278 μg/g (dry weight) in krill exoskeleton, and 178−285 μg/g (dry weight) in krill muscle.[12] Our preliminary study indicated a fluoride concentration in whole frozen krill of 220−240 μg/g (wet weight). A high level of fluoride is toxic for human consumption. Chronic fluoride ingestion of 1.7 μg/mL fluoride in drinking water can cause mottling of the teeth in 30−50% of humans, and chronic fluorosis may cause osteosclerosis, calcification of ligaments and tendons, bony exostoses, and renal calculi.[13] Prior research showed an alkali solubilization and acid precipitation method was suitable for recovering proteins from Antarctic krill. However, the fluoride concentration of the extracted proteins needs to be decreased. Therefore, extracting proteins with low fluoride level from Antarctic krill was investigated in this work. In addition, the extract composition was analyzed.

MATERIALS AND METHODS

Materials. Frozen Antarctic krill (*E. superba*) squeezed into blocks was provided by Japan Fisheries Co., Ltd. They were crushed by a hammer crusher in a low-temperature warehouse. The crushed krill was stored at −30 °C until use. All of the chemical agents were of analytical grade.

Effect of pH on Protein Solubilization. Distilled water was added to frozen krill at a ratio of 6 (water/krill, mL/g). The mixture was stirred until the frozen krill block disappeared and then thoroughly homogenized for 1 min using an Ultra-Turrax homogenizer (type T 45, Jahnke & Kunkel, Stauffen). The homogenates were separately adjusted

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to basic pH (8.5, 9.5, 10.5, 11.5, and 12.5). NaOH was used for pH adjustment. Once the desired pH was reached, the mixture was stirred for 30 min at 4 °C to solubilize krill proteins, followed by centrifugation at 4500*g* for 10 min. The supernatant was collected for analyses of dissolved protein rate (DPR) and dissolved fluoride rate (DFR), respectively.

DPR was calculated by determining the protein content of supernatant as a percentage of that of material. DFR was calculated by determining the fluoride content of supernatant as a percentage of that of material.

Solubilization of Krill Proteins. To improve the solubilization of krill proteins, two protein solubilizations were performed. In the first protein solubilization, the homogenate was adjusted to pH 11.5 and the mixtures were stirred for different durations (30, 60, and 90 min) at 4 °C. After centrifugation, the supernatant and krill residues were collected. The first supernatant was used for analyses of DPR and DFR.

The residues after first protein solubilization for 30 min were used for the second protein solubilization. Distilled water was added to the krill residues at different ratios (2, 3, 4, water/krill residue, mL/g), and then the mixture was adjusted to pH 11.5, stirred for 30 min for protein solubilization, and finally centrifuged to obtain the supernatant. The first and second supernatants were mixed for analyses of DPR and DFR, respectively.

Acid Precipitation of Krill Proteins. The supernatant obtained by the optimum method for protein solubilization was employed to investigate acid-induced protein precipitation. The liquor was precisely adjusted to different pH values (3.5, 4.0, 4.4, 4.5, 4.6, 4.7, 4.8, 5.0, 5.5) by HCl. Once the desired pH was reached, the reaction was allowed to take place for 20 min at 4 °C, followed by centrifugation at 4500*g* for 10 min. The supernatants were collected, in which the protein content was analyzed.

The optimum pH of protein precipitation was determined by the rate of protein content in the supernatant to that in the material, at which the rate was the lowest value. The precipitated proteins at the optimum pH were collected, in which moisture and fluoride contents were analyzed.

Fluoride Removal from Precipitated Proteins. The fluoride removal from precipitated proteins at optimum pH was investigated. Our preliminary experiments (data not shown) showed that the fluoride in frozen krill was easily solubilized at acid conditions, so we deduced that the fluoride could be removed from the precipitated proteins by washing at acid conditions before the proteins were precipitated at the optimum pH. Through the acid-induced protein precipitation at pH 4.6, the precipitated proteins without washing were obtained. Distilled water was added to the precipitated proteins at different ratios (2, 3, 4 mL/g). The mixture was precisely adjusted to pH 4.6 by 2 M HCl. Once the desired pH was obtained, the mixture was centrifuged at 4500*g* for 10 min. The process was called one washing. Through one washing, the first precipitated proteins (FPP) by fluoride removal were obtained. Distilled water was added to the FPP at different ratios (2, 3, 4 mL/g), and the pH adjustment and centrifugation used in one washing were followed. This process was called two washings. Through two washings, the second precipitated protein (SPP) by fluoride removal was obtained. Distilled water was added to SPP at different ratios (2, 3, 4 mL/g). Through repetition of the process of pH adjustment and centrifugation as shown in one washing, three washings were completed and the third precipitated proteins (TPP) by fluoride removal were obtained.

Fluoride contents in FPP, SPP, and TPP were analyzed, respectively. The precipitated proteins obtained by the optimum fluoride removal were called LFP. Protein yield was analyzed, which was calculated by determining the protein content of LFP as a percentage of that of krill.

Composition Analysis of LFP. For investigating the nutritional value of LFP, the essential amino acids, nonessential amino acids, and fatty acids were analyzed.

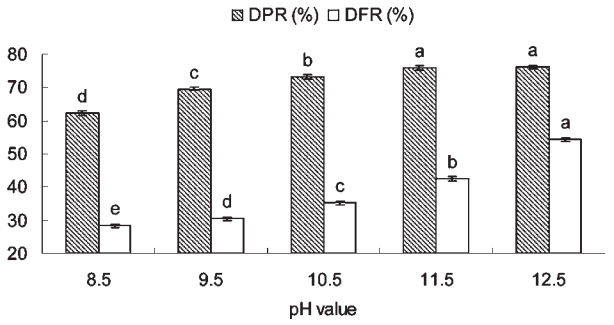

Figure 1. Effect of pH on krill protein solubilization. DPR, dissolved protein rate; DFR, dissolved fluoride rate. The results at each pH value with different letters are significantly different ($P < 0.05$).

Chemical Analysis. Fluoride content was determined by the standard fluoride selective ion electrode method. Moisture content was determined by the constant weight method at 105 °C. Protein content (N × 6.25) was determined by the Kjeldahl method. Total lipids were determined by the classic "Folch" method,[14] in which a ratio of 1 part of sample to 20 parts of chloroform/methanol (2:1) was employed, followed by washing of the crude extract. The extracted lipids were preserved at −35 °C for the further analyses of total phospholipids and fatty acids. Total phospholipids were estimated by measuring phosphorus in the total lipids using the AOCS standard method. Fatty acid analysis of the extracted total lipids was performed according to the method of Wang and Xue.[15]

Analyses of essential and nonessential amino acids were conducted according to AOAC method 982.30 E(a, b, c). The freeze-dried LFP was hydrolyzed with the following three types of hydrolysis: acid hydrolysis with 6 M HCl at 110 °C for 24 h; performic acid oxidation at 5 °C overnight followed by acid hydrolysis (6 M HCl at 110 °C for 24 h); and alkaline hydrolysis with 4.2 M NaOH at 110 °C for 22 h. After hydrolysis, amino acids were quantified by an automatic amino acid analyzer (Waters M 510), using a PICO.TAG column.

Data Analysis. Values were expressed as the mean ± standard deviation of three parallel measurements. Differences between variables were tested for significance by one-way ANOVA using SPSS 11 (SPSS Inc., Chicago, IL). A difference was considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Effect of pH on Protein Solubilization. The effect of pH on krill protein solubilization is shown in Figure 1. DPR increased with the increase of pH value, and a similar tendency was observed for DFR. However, the improvement of DPR was not significant ($P > 0.05$) when the pH value was >11.5. Meanwhile, the abrupt improvement of FDP was significant ($P < 0.05$). To reduce the fluoride content of terminal proteins, it is necessary to reduce DFR and increase DPR during the protein solubilization. Proteins extracted at a higher pH may undergo undesirable chemical modifications, including protein denaturation and chemical change. For many kinds of proteins, such as isolated soybean proteins, the pH range for alkaline solubilization is between pH 7.5 and 9.0 in practice. However, the crude enzymes extracted from frozen Antarctic krill had higher protease activities when the pH was between 8 and 11,[16] which was not beneficial to the protein precipitation at the following steps. Therefore, the optimum pH value for protein solubilization was set as 11.5, which was the same pH value used by Gigliotti et al.[11]

Table 1. DPR and DFR of the Supernatant Liquors Obtained by Different Methods[a]

method of protein solubilization	DPR (%)	DFR (%)
A	76.90 ± 0.57 c	42.60 ± 0.61 d
B	77.62 ± 0.58 c	46.86 ± 0.42 c
C	78.54 ± 0.55 c	47.98 ± 0.49 bc
D	92.39 ± 0.57 b	48.41 ± 0.59 bc
E	97.32 ± 0.59 a	48.78 ± 0.40 b
F	98.81 ± 0.49 a	51.27 ± 0.68 a

[a] Results in a column with the same letter are insignificantly different ($P > 0.05$). Methods of protein solubilization are described in detail under Materials and Methods: A, the mixture was stirred for 30 min during one protein solubilization; B, the mixture was stirred for 60 min during one protein solubilization; C, the mixture were stirred for 90 min during one protein solubilization; D, distilled water was added to the krill residues at the ratio of 2 during two protein solubilizations; E, distilled water was added to the krill residues at the ratio of 3 during two protein solubilizations; F, distilled water was added to the krill residues at the ratio of 4 during two protein solubilizations. DPR, dissolved protein rate; DFR, dissolved fluoride rate.

Solubilization of Krill Proteins. Different methods were employed to solubilize krill proteins, and the results are shown in Table 1. For one protein solubilization, DPR increased with the increase of time, and it was the same for DFR. Krill proteins can be solubilized in alkaline solutions due to the interactions between charged protein molecules and dipolar water molecules. When the extraction time was 30 min, the krill protein solubility nearly reached saturation level. This was why the improvement of DPR was not significant ($P > 0.05$) when the time increased from 30 to 90 min (shown in Table 1). Thus, 30 min was the optimum time for one protein solubilization. Compared with the DPR of one protein solubilization, the DPR of two protein solubilizations showed a significant increase ($P < 0.05$). For two protein solubilizations, DPR increased with the increase of the ratio of water to krill residue during the second protein solubilization. A similar result was also observed for DFR. Although DPR with a rate of 4 showed a slight increase (not significant, $P > 0.05$) compared to that of 3, DFR with a rate of 4 exhibited a significant increase ($P < 0.05$) compared to that of 3. High DFR led to a high fluoride content in the precipitated proteins obtained in the sequential steps. Moreover, DPR with a rate of 3 reached 97.32%, which was beneficial to the following protein precipitation. Therefore, two protein solubilizations were necessary, and the optimum ratio of water to krill residue was 3 during the second solubilization.

Acid Precipitation of Krill Proteins. Figure 2 shows the effect of pH on protein precipitation. The rate reached the minimum at pH 4.6, and lower or higher than pH 4.6 would lead to a significantly ($P < 0.05$) increased rate, which indicated 4.6 was the optimum pH value of protein precipitation. A pH value of 5.5 has been used to precipitate proteins to recover them from Antarctic krill,[4,17] but this work showed pH 4.6 was the optimum value. Most proteins are negatively charged at neutral pH and generally have isoelectric points (pI) between 4 and 6. Antarctic krill proteins consisted of different kinds of proteins: acid-precipitated proteins, alkali-precipitated proteins, and unprecipitable proteins. Therefore, 38.44% crude protein of krill raw materials could not be precipitated at pH 4.6.

Moisture and fluoride contents of the precipitated proteins were 82.26 ± 0.26% on a wet weight basis and 106.62 ± 0.18 mg/kg on a dry weight basis, respectively. According to the Chinese National Standard for maximum levels of contaminations in food (GB 2762-2005), the maximum level of fluoride content of freshwater fish was 2 mg/kg. Therefore, the precipitated proteins could not be used directly for human food due to its high fluoride content. Thus, the fluoride needed be removed from the precipitated proteins.

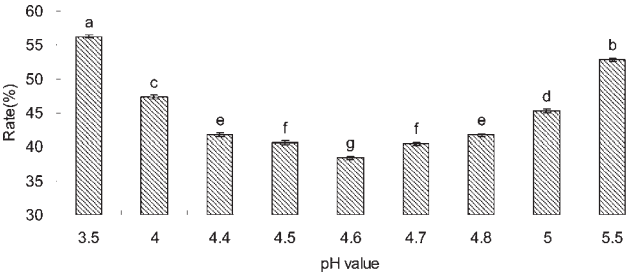

Figure 2. Effect of pH on krill protein precipitation. The results at each pH value with different letters are significantly different ($P < 0.05$).

Table 2. Fluoride Content (Milligrams per Kilogram on a Dry Weight Basis) of the Precipitated Proteins Obtained by Different Methods[a]

method of fluoride removal	FPP	SPP	TPP
A	35.75 ± 0.15 a	15.16 ± 0.14 a	9.83 ± 0.17 a
B	33.25 ± 0.11 b	9.86 ± 0.15 b	5.76 ± 0.16 b
C	32.90 ± 0.18 b	9.79 ± 0.13 b	5.72 ± 0.14 b

[a] Results in a column with the same letter are insignificantly different ($P > 0.05$). A, distilled water was added to the precipitated proteins at the ratio of 2 during fluoride removal of precipitated proteins; B, distilled water was added to the precipitated proteins at the ratio of 3 during fluoride removal of precipitated proteins; C, distilled water was added to the precipitated proteins at the ratio of 4 during fluoride removal of precipitated proteins. FPP, precipitated proteins after one washing; SPP, precipitated proteins after two washings; TPP, precipitated proteins after three washings.

Fluoride Removal from Precipitated Proteins. The fluoride content of the precipitated proteins without washing was 106.62 mg/kg (dry weight). The fluoride contents of the precipitated proteins obtained by different methods of fluoride removal are shown in Table 2. Compared to the sample without washing, the sample after washing showed a significant ($P < 0.05$) decrease in fluoride content. Meanwhile, the fluoride content decreased with the increase of rate of water to the precipitated proteins, but the decrease was not significant ($P > 0.05$) when the rate was >3. On the basis of the effect of both fluoride removal and water consumption, the rate of 3 was optimum for removing fluoride from the precipitated proteins. At the same rate of water to precipitated proteins, the fluoride content decreased with the increase of washings. After two washings at the rate of 3, the fluoride content of precipitated proteins reached 9.86 mg/kg (dry weight), which was approximately 1.55 mg/kg (wet weight) because the moisture content of the precipitated proteins was 84.23% (shown in Table 3). According to U.S. FDA regulations, 2.4 μg/mL fluoride in bottled water and beverages is permitted. Therefore, through two washings at the rate of 3, LFP could be obtained and the fluoride content in the LFP was safe when it was used for human food. Our results showed the washing method was very effective in removing fluoride from the extracted proteins.

Table 3. Basic Composition and Fatty Acid Composition of LFP[a]

composition measurement	LFP
basic composition	
moisture (%)	84.23 ± 0.18
crude protein (%, dry weight)	66.96 ± 0.16
total lipids (%, dry weight)	33.01 ± 0.80
total phospholipids (mg/g total lipids)	385.67 ± 0.32
PUFAs (%)	
EPA 20:5 (n-3)	13.65 ± 0.01
DHA 22:6 (n-3)	7.17 ± 0.01
MUFAs (%)	
16:1	8.70 ± 0.01
18:1	20.61 ± 0.02
SFAs (%)	
14:0	12.67 ± 0.02
16:0	24.65 ± 0.03
18:0	1.60 ± 0.02

[a] LFP, proteins with low fluoride level; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

Table 4. Amino Acid Composition of LFP[a]

amino acid	LFP (mg/g protein)	FAO/WHO/UNU (2007) adult (infant) (mg/g protein)
essential amino acids		
histidine	16.6 ± 0.2	15 (16)
isoleucine	53.0 ± 0.1	30 (31)
leucine	76.2 ± 0.2	59 (61)
lysine	59.8 ± 0.1	45 (48)
methionine + cysteine	69.9 ± 0.4	22 (24)
phenylalanine + tyrosine	86.3 ± 0.4	38 (41)
threonine	49.8 ± 0.2	23 (25)
tryptophan	15.8 ± 0.1	6 (6.6)
valine	88.2 ± 0.1	39 (40)
total EAA	515.6	277 (292.6)
nonessential amino acids		
cysteine	32.9 ± 0.3	
tyrosine	38.4 ± 0.1	
glutamate	136.7 ± 0.4	
aspartate	107.4 ± 0.3	
alanine	68.7 ± 0.4	
arginine	47.3 ± 0.1	
serine	42.2 ± 0.2	
glycine	36.6 ± 0.2	
proline	35.2 ± 0.3	
total NEAA	545.4	

[a] LFP, proteins with low fluoride level; total EAA, total essential amino acids; total NEAA, total nonessential amino acids.

DPR of Antarctic krill was 97.32%. Part of the Antarctic krill proteins (38.44%) could not be precipitated at pH 4.6. That meant approximately 58.88% Antarctic krill proteins could be precipitated. Through fluoride removal from the precipitated proteins, the protein recovered from Antarctic krill resulted in LFP with a protein recovery yield of 52.68 ± 0.52%. Therefore, approximately 6.20% of Antarctic krill proteins were lost during fluoride removal, which was due to the dissolvation in the centrifuged supernatant. However, processing of 100 kg of raw fish by surimi technology can obtain 19.5 kg of proteins, and a lot of proteins are lost in surimi wash water.[18] Therefore, the protein yield of 52.68% is much higher than that of surimi processing. Chen et al.[4] reported the protein yield was 45−50% (dry weight) during krill protein concentration recovered from Antarctic krill by alkali solubilization and acid precipitation. The reason for the difference was the extracting techniques and calculation methods.

Composition Analysis of LFP. Table 3 shows the composition of LFP, including 84.23% of moisture, 66.96% of proteins (dry weight basis), and 33.01% of total lipids (dry weight basis). Total phospholipids were 385.67 mg/g total lipids. The values of total lipids and total phospholipids were relatively high. The phospholipids in *E. superba* are >40% of total lipids,[19] and about 65% of fatty acids in krill are incorporated into phospholipids.[20] Due to their amphiphilic characteristics, phospholipids can act as an emulsifier, which allows them to undergo interactions with apolar triglycerides and charged molecules such as proteins. The krill protein molecules were unfolding during protein solubilization, which increased their surface hydrophobicity because of exposure of hydrophobic regions that were buried in the protein interior. This increased hydrophobicity, resulting in the formation of hydrophobic interactions between krill proteins and triglycerides along with the phospholipids. Therefore, during the following centrifugation, the insoluble substances (e.g., shell) were precipitated and most of the lipids were retained by the solubilized protein phase. As the krill proteins were refolded at pH 4.6 during the acid precipitation, the lipids were probably trapped in the interior of the refolding proteins due to interactions between the protein's hydrophobic regions and the lipids that were formed during the alkali solubilization. Subsequently, the krill proteins and most of the lipids were separated by the liquid/solid centrifuge. During the fluoride removal of precipitated proteins, the interactions between the proteins and lipids still existed. Therefore, the lipid retention in the recovered LFP was high.

Table 3 shows that LFP lipids contained 21% polyunsaturated fatty acids (PUFAs), 29% monounsaturated fatty acids (MUFAs), and 39% saturated fatty acids (SFAs). Fatty acids of LFP appeared to be rich in EPA (13.65%) and DHA (7.17%), which was important because EPA and DHA were linked to various health benefits.[21] One of the major drawbacks of products containing a high amount of unsaturated fatty acids is their high susceptibility to oxidation, which involves the formation of toxic products such as peroxides or volatile compounds relative to undesirable off-flavors. Therefore, a further investigation of the oxidative stability of LFP is needed.

The amino acid content of LFP was compared to the WHO/FAO/UNU[22] amino acid requirements for adults and infants (Table 4). Glutamic acid and aspartic acid were the most abundant amino acids in LFP, making up about 136.7 and 107.4 mg/g protein, respectively. The total essential amino acids for LFP were 515.6 mg/g protein, which exceeded 277 (292.6) mg/g protein for the essential amino acid requirements of adults (infants). LFP contained all nine essential amino acids, and each was sufficient in amount to meet FAO/WHO/UNU requirements for adults (infants). Therefore, the LFP recovered from krill may be a useful additive in the development of high-quality food products that require a high content of essential amino

acids. However, protein quality is determined not only by the essential amino acid composition but also by its digestibility. On the basis of an animal feeding study, krill protein concentrate recovered from Antarctic krill (*E. superba*) by alkali solubilization and acid precipitation is comparable to casein for digestibility, PER, and PDCAAS scores.[11]

In conclusion, the optimum pH value for protein solubilization was determined to be 11.5. Two protein solubilizations were necessary. The optimum pH value for protein precipitation was 4.6. LFP with a fluoride content of 9.86 mg/kg (dry weight) was obtained after fluoride removal. The protein yield of LFP was 52.68%. Composition analysis of the LFP showed it contained 66.96% of proteins and 33.01% of total lipids on a dry weight basis. The LFP contained all nine essential amino acids in sufficient amounts to meet FAO/WHO/UNU requirements for adults and infants and was rich in EPA and DHA. Further studies are needed to investigate the oxidative stability and bioavailability of LFP.

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ABBREVIATIONS USED

BV, biological value; DPR, dissolved protein rate; DFR, dissolved fluoride rate; FPP, first precipitated proteins; SPP, second precipitated proteins; TPP, third precipitated proteins; LFP, proteins with low fluoride level; pI, isoelectric points; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids; EAA, essential amino acids; NEAA, nonessential amino acids.

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