Recovery of Bioavailable Calcium from Alaska Pollack (*Theragra chalcogramma*) Fish Backbone By-products by Pepsinolytic Hydrolysis

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Abstract

Fish backbone, a major by-product in the fish processing industry, accounts for about 15% of whole fish weight. In this study, recovery of bioavailable calcium from Alaska pollack (*Theragra chalcogramma*) backbone by-products using enzymatic hydrolysis was investigated. Finely ground fish backbones were hydrolyzed with two proteolytic enzymes (pepsin and protease) to obtain soluble calcium from the by-products. The pepsin digest had a higher degradation efficiency (88%) than protease. Four different concentrations of the fish backbone calcium (100, 250, 500 and 1000 mg/L) prepared by the pepsin digest were treated with Na_2HPO_4 at a concentration gradient (0, 1, 2, 4, 8, 10, 15 and 20 mM) to evaluate their solubility, revealing that solubilities of the fish backbone calcium were superior to those of $CaCl_2$ at all the calcium and Na_2HPO_4 concentrations. Among the tested concentrations the highest solubility was found in the pepsin digest containing a calcium concentration of 1000 mg/L. Thus, hydrolyzing with pepsin is an effective mode of recovering bioavailable calcium from Alaska pollack fish backbones.

Key words: fish backbone by-product, bioavailable calcium, solubility, pepsinolytic hydrolysis, enzymatic hydrolysis

INTRODUCTION

The domestic fish industry creates a large amount of waste or by-products with high nutrient content. If such waste is not properly utilized in human or animal nutrition, it has to be deposited to the environment, which creates pollution problems. Despite international attempts to decrease waste through various kinds of waste treatment systems, the quantity of the waste produced has been increasing annually (1). Due to such problems, there has been an increasing interest recently in fish by-products as an under-utilized resource.

During the last decades, the annual world fish production has stabilized at about 90 million tons, and according to experts, no further growth is expected in the future. However, only 50~60% of the production is used for human consumption and the rest has been discarded as by-products. Removal of fish skin and bones is necessary for preparing fish fillet, minced fish and Sashimi. The backbone is one of the major fractions of fish waste, accounting for 15% of the fish weight. It contains 30% protein and 60~70% minerals, especially calcium, one of the most essential minerals in the human

body (2). Although the nutritional value of these wastes is high, no substantial attempt is being made to utilize these potentially valuable resources.

Fish by-products can be categorised in different ways such as intestines, skin, bones and scales. Currently, some are used in animal feeds and fertilizers. The major fraction of the by-products is used for feed production such as fish meal and oil, and most of the remainder is used in fertilizer because it improves crop yield. However, these uses have low profitability. Producing products for human consumption can achieve a better profitability; especially by producing bioactive compounds (extracting and purifying) such as enzymes, bioactive peptides, and biopolymers for biotechnological or pharmaceutical application. The by-products contain valuable protein and lipid fractions in addition to minerals and vitamins.

Calcium is one of the major structural elements in bones and teeth. Approximately 2% of the total human body weight (about 1,200 g) is calcium. Calcium is distributed throughout the body, but 99% of it is concentrated in bones and teeth. The remaining 1% is used in nerve impulse transmission, muscle contraction, axonal flow, cytoplasmic streaming, chromosome movement,

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neurotransmitter release, endocytosis and exocytosis blood clotting, secretion of hormones, hormone functioning such as insulin, membrane ion transport and heart functioning and mediation of the constriction and relaxation of blood vessels (3,4). Some of the major symptoms of calcium deficiency are skeletal abnormalities, osteopenia, osteomalacia, osteoporosis and rickets. Other symptoms of calcium deficiency are insomnia, tetany, premenstrual cramps and hypertension (high blood pressure). Furthermore, low calcium intakes have also been linked to premature births and some forms of cancer including colon and breast cancer.

Many studies have been carried out to utilize proteins, oils, minerals, carbohydrates and nucleic acids deriving from fishery by-products, and to develop their functional properties (1,5-7). However, few studies have been reported so far on the recovery of fish bone calcium for human nutrition (8,9). Previous studies have compared similarities and differences between fish bone and mammalian bone, and have also defined the structure, distribution, function and activity of organic components in fish bone (10,11). These studies suggest that wasted fish bones may have the potential to be a good source of bioavailable calcium that could be extracted and used as a nutritional supplement. The bioavailability depends on absorbability and the incorporation of absorbed calcium into bone. In addition, calcium forms biologically unavailable compounds with many of the anions present in food such as carbonate, oxalate and phosphate. The effect of these anions is to decrease the efficiency of calcium absorption. Free ionic calcium (Ca^{2+}) or calciums bound to soluble organic molecules are the physiologically active form of calcium and all forms of this mineral are broken down to their ionic form before absorption (12).

Hence, the objective of this study was to recover bioavailable calcium from Alaska pollack (*Theragra chalcogramma*) backbones by enzymatic hydrolysis of the by-products and to evaluate its solubility in presence of Na_2HPO_4 which can bind calcium to form a precipitate and then protect its absorbability into the human body.

MATERIALS AND METHODS

Materials

Enzymes (pepsin and protease) were purchased from Sigma Co. (St. Louis, USA). Alaska pollack fish backbones was kindly donated from Daerim Co. (Busan, Korea), and rinsed with distilled water before freeze-drying and stored at -70°C until use. All the other chemicals used were of analytical grade available commercially.

Proximate chemical composition

A proximate chemical composition of Alaska pollack fish backbone was determined according to the AOAC methods (13). Crude lipid content was determined by Soxhlet extraction and crude protein content was determined by the Kjeldhal method. Ash content was determined by calcination in a furnace at 550°C and the moisture content was determined by drying in an oven at 105°C for 24 hr.

Enzymatic hydrolysis

Prior to enzymatic treatment, freeze-dried Alaska pollack backbones were crushed into powder. The enzymatic reaction mixture was prepared by adding 100 mL of buffer solution with pH 2.0 (pepsin) and pH 2.8 (protease) to 1 g of freeze-dried Alaska pollack backbones. The mixtures were then initially pre-incubated and digested with pepsin and protease at 37°C for 24 hr (Enzyme-to-substrate ratio [E/S]: 1/1,000, 2.5/1,000, 5/1,000, 7.5/1,000 and 1/100 or 0.1, 0.25, 0.5, 0.75 and 1%, respectively). The hydrolysis was stopped by heating the enzymatic reaction solutions at 100°C for 15 min to inactivate the enzymes. The reaction solutions were allowed to cool and centrifuged at 3,000 g for 20 min and freeze-dried. Protein and calcium concentrations were measured to evaluate protein and calcium recovery rates. The degradation efficiency (%) was calculated as the ratio of degraded weight of substrate to its initial weight as reported previously (14).

Amino acid analysis

The hydrolysates were incubated for 24 hr in 6 N HCl at 110°C in vacuum-sealed ampoules. After neutralizing, evaporating, and filtering; the amino acid composition was determined with an amino acid analyzer (Biochrom 20, Pharmacia LKB Biochrom, UK).

Determination of protein and calcium contents

For the determination of protein content, trichloroacetic acid (3 mL, 5%) and phosphate buffer (1 mL, pH 8.0) were added to the sample (1 mL) and after 15 min at room temperature, the precipitate was removed by centrifugation at 3,000 g for 15 min.

The protein content was then determined by the Lowry-Folin method (15) using BSA as the standard. Calcium content was determined by using the Compact Inolab pH/ION level 2, precision measuring instrument (Wissenschaftlich Technische Co, Weilheim, Germany).

Binding of fish backbone calcium with peptides in presence of Na_2HPO_4

The potential of fish backbone calcium from pepsin digest to form insoluble calcium precipitate in presence

of disodium hydrogen phosphate (Na_2HPO_4) was investigated. From the pepsin digest, four different calcium concentrations equivalent to 100, 250, 500 and 1,000 mg/L were prepared as mentioned below. Pepsin digest was poured into a beaker and respective calcium concentrations were adjusted with distilled water by using the Compact Inolab pH/ION level 2 calcium selective probe instrument. Thereafter, for the eight portions of each concentration of pepsin digests, Na_2HPO_4 was added (0, 1, 2, 4, 8, 10, 15 and 20 mM, each) and stirred at room temperature. After 20 min, the mixtures were centrifuged and the soluble calcium content was determined by assaying the calcium content of the solution. A calcium chloride ($CaCl_2$) solution, under similar conditions, was treated as a control for the comparison.

Statistical analysis

Statistical analyses were conducted with the SPSS 11.5 version software package on triplicate (n=3) test data. The mean values of each treatment were compared using one-way analysis of variance (ANOVA) followed by Turkey test. The p-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Proximate chemical composition

The proximate composition of freeze-dried Alaska pollack fish backbone was: moisture 5.1%, ash content 40.5%, protein 50.5% and lipid 4.4%.

Enzymatic hydrolysis

Although collagenase is a well-characterized enzyme for collagen degradation, for practical considerations, commercially available enzymes were used. In this study pepsin and protease which exert optimal activity in a moderately strong acidic range of pH 2 were evaluated for their effectiveness for degradation of Alaska pollack backbone. The enzymatic hydrolysis was carried out at five different [E/S] ratios in order to determine the correlation between the degradation efficiency and [E/S] ratios. According to Fig. 1, when the [E/S] ratio increased to 0.75%, the degradation efficiency with pepsin increased to 88% and thereafter, it declined. Protease digestion resulted in a significant difference (p<0.05) in degradation efficiency at increased [E/S] ratios. At 0.1% [E/S] ratio of protease, 68% of degradation efficiency was observed and 1% showed 76% of degradation efficiency. However, no correlation was observed between degradation efficiency and [E/S] ratios.

The yields of hydrolyzed protein and calcium content after application of the different concentrations of pepsin and protease are shown in Fig. 2. As shown in Fig. 2a,
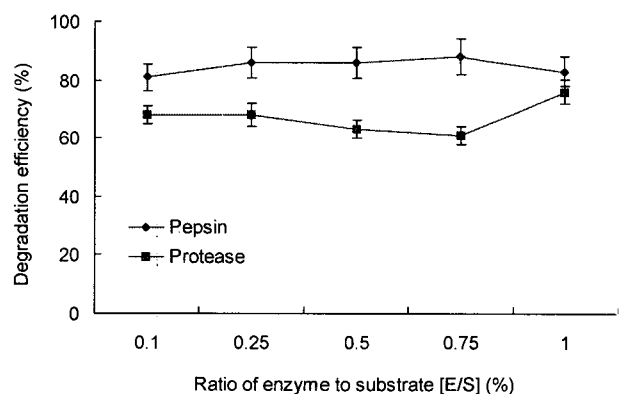

Fig. 1. Degradation efficiency of Alaska pollack fish bone by protease and pepsin. All data were expressed as mean values (mean ± SD, n=3).
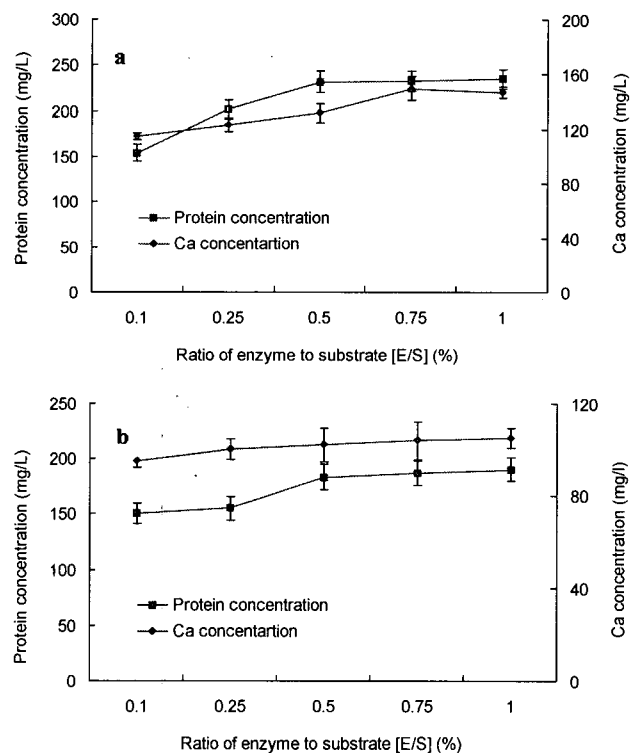

Fig. 2. Calcium and protein concentrations in (a) pepsinolytic and (b) proteolytic digests of fish backbone. All data were expressed as mean values (mean ± SD, n=3).

after the pepsin ratio increased to 0.5 and 0.75%, the yield of protein and calcium concentrations increased (154 to 232 and 115 to 150 mg/L, respectively) significantly (p<0.05). A positive correlation was shown between protein (r^2 = 0.7) and calcium (r^2 = 0.9) concentrations with the increment of [E/S] ratio. According to Fig. 2b, protein (r^2 = 0.9) and calcium (r^2 = 0.9) concentrations were positively correlated with the increment of protease dosage. However, the yields of protein and calcium were less than those of the pepsin digest. Thus, it was clear that pepsin can more effectively degrade

fish backbone and release soluble calcium than the protease tested. Therefore, pepsinolytic hydrolysis is a very important process for optimum utilization of fish backbone calcium.

In a previous study, it was found that the organic portion of hoki fishbone (*Johnius belengerii*) mainly consisted of protein (28.0%), lipid (1.94%), and carbohydrate (0.56%), and the inorganic portion, the minerals calcium (59.7%) and phosphorus (35.8%). The inorganic portion is mainly composed of hydroxyapaptite crystals deposited within organic matrices of cross-linked collagen fibrils. Hence, to obtain soluble calcium from fish bone tissue, hydroxyapaptite crystals released by breaking down the cross-linked structure of the organic matrix should be dissolved (9). It can be concluded from the present study that pepsinolytic hydrolysis has significant efficacy for the degradation of Alaska pollack fish back bone to protein and calcium.

Pepsin is one of the gastric proteases of vertebrates which are active under acidic conditions (pH<5) (16). The precursor of pepsin is pepsinogen, which is converted to pepsin in the presence of hydrochloric acid. Pepsin is a globular endopeptidase, which hydrolyses at peptide bonds formed by aromatic amino acids or dicarboxylic amino acids. Its action breaks long polypeptide chains into shorter lengths. When compared with acid or alkali hydrolysis, the hydrolysis of protein using selective protease enzymes can be a much more efficient process. Also, because of using enzymes, it gives few or no undesirable side reactions or products. In addition, the final digest contains less salt after neutralization and the functionality of the final product can be governed by selection of specific enzymes and reaction factors (17, 18), and this hydrolyzed protein can be used for human consumption. Furthermore, enzymatic modification of proteins using selective proteases to split specific peptide bonds is widely used in food industry. These peptides have a smaller molecular size than that of proteins. Therefore, their functional properties are also changed, and the functional properties of hydrolyzed proteins are primarily determined by molecular size and hydrophobic characters (19).

Amino acid analysis of pepsinolytic hydrolysate of Alaska pollack

The amino acid composition of the hydrolysates consisted mostly of aspartic acid, glutamic acid, arginine, glycine, threonine, proline, alanine, serine, and lysine, which have the ability to bind calcium (Table 1). A high concentration of glutamic acid, aspartic acid and arginine residues was observed in fish bone protein, and were 13.2%, 10.4% and 10.3%, respectively, of the total

Table 1. Amino acid compositions of Alaska pollack fish backbone hydrolysates liberated by pepsin digestion

Amino acid	mg/100 g
Asp	5300.34
Thr	2209.09
Ser	2614.00
Glu	6770.63
Pro	4535.55
Gly	2177.27
Ala	2526.91
Cys	972.45
Val	2176.55
Met	595.62
Ile	2456.64
Leu	3695.33
Tyr	1833.65
Phe	2954.58
His	1768.03
Lys	2861.46
Arg	5261.47
Total	50709.57

All data were expressed as mean values (n=3).

amino acids. These amino acids accounting for 36.9% of total amino acid content might contribute to calcium binding property of the pepsinolytic digest of Alaska pollack. Aspartic acid and glutamic acid maintain the solubility and ionic character of proteins (free carboxyl group makes it acidic and hydrophilic). The negatively charged side chains of these amino acids function as the binding sites for minerals and enhance calcium-binding capacity and inhibit the formation of insoluble calcium phosphate. Aspartic acid also functions as a transport mechanism, delivering the calcium to proper body sites for specific functions (20). This form of calcium is readily absorbed throughout the body and released to blood to be easily absorbed, assimilated and utilized.

Casein phosphopeptides, a negative charged group which are representative of peptides possessing, are soluble in the presence of calcium and lead to better absorption, assimilation and usage in the body. Ca-binding phosphoproteins include osteocalcin, phosvitin and casein phosphoprotein with binding sites at serine, threonine, alanine and tyrosine residues (21). Furthermore, proline, glycine, and hydroxyproline residues are typical amino acids of collagen, which is a connective protein in the bones (22). In addition, Jung et al. (9) reported that the amino acid composition in hoki bone hydrolysate obtained by tuna intestine crude enzyme digestion, consisted of glycine, threonine, glutamine, glutamic acid, alanine, aspartic acid and asperagine, serine, hydroxyproline, and arginine. As reported by Nishimoto et al. (10), osteocalcin from carp, *Cyprinus carpio*, has high ratios of alanine, tyrosine, glutamine and aspartic

acid. In contrast, the calcium-binding phospopeptides obtained from hen egg yolk mainly consists of serine, aspartic acid, asparagines, glutamine, glutamic acid, glysine, arginine, alanine and lysine (23). Oligophosphopeptide from egg yolk consisted of serine, aspartic acid, asparagines, glutamic acid, glutamine and arginine; and the phosphoseryl group in the oligophosphopeptide is important in calcium binding (23). Thus, the amino acid composition of Alaska pollack fish backbone protein suggests that it has a high affinity to bind with calcium.

Binding of fish backbone calcium with peptides in the presence of Na₂HPO₄

Pepsin digest containing bone calcium was treated with different concentrations of Na₂HPO₄ and thereafter precipitation of calcium with phosphate ions was compared with CaCl₂ solution as a control at similar conditions (alkali conditions). According to Fig. 3a and Fig. 3b, the soluble calcium concentrations in both of the

digest and control were significantly and concentration-dependently decreased ($p < 0.05$) showing the same correlation ($r^2 = 0.9$) with Na₂HPO₄ concentration. Since the soluble calcium concentrations were consistently higher in the pepsin digests, it can be assumed that phosphopeptides protected against calcium binding with Na₂HPO₄. Nevertheless, much of fishbone calcium was combined with phosphate of Na₂HPO₄ to form the calcium phosphate precipitate. However, as depicted in Fig. 3c, calcium concentration was not reduced (415 to 400 mg/L) significantly ($p < 0.05$) up to 10 mM of Na₂HPO₄, but above of this concentration, a decrease could be observed. In addition, the fish bone calcium precipitation ($r^2 = 0.7$) exhibited less correlation than CaCl₂ ($r^2 = 0.9$) with Na₂HPO₄, because fish bone calcium and phosphopeptide content were increased against Na₂HPO₄ demonstrating the affinity to bind calcium with phosphopeptides. As shown in Fig. 3d, the fish bone calcium concentration equivalent at 1000 mg/L remained constant and did not
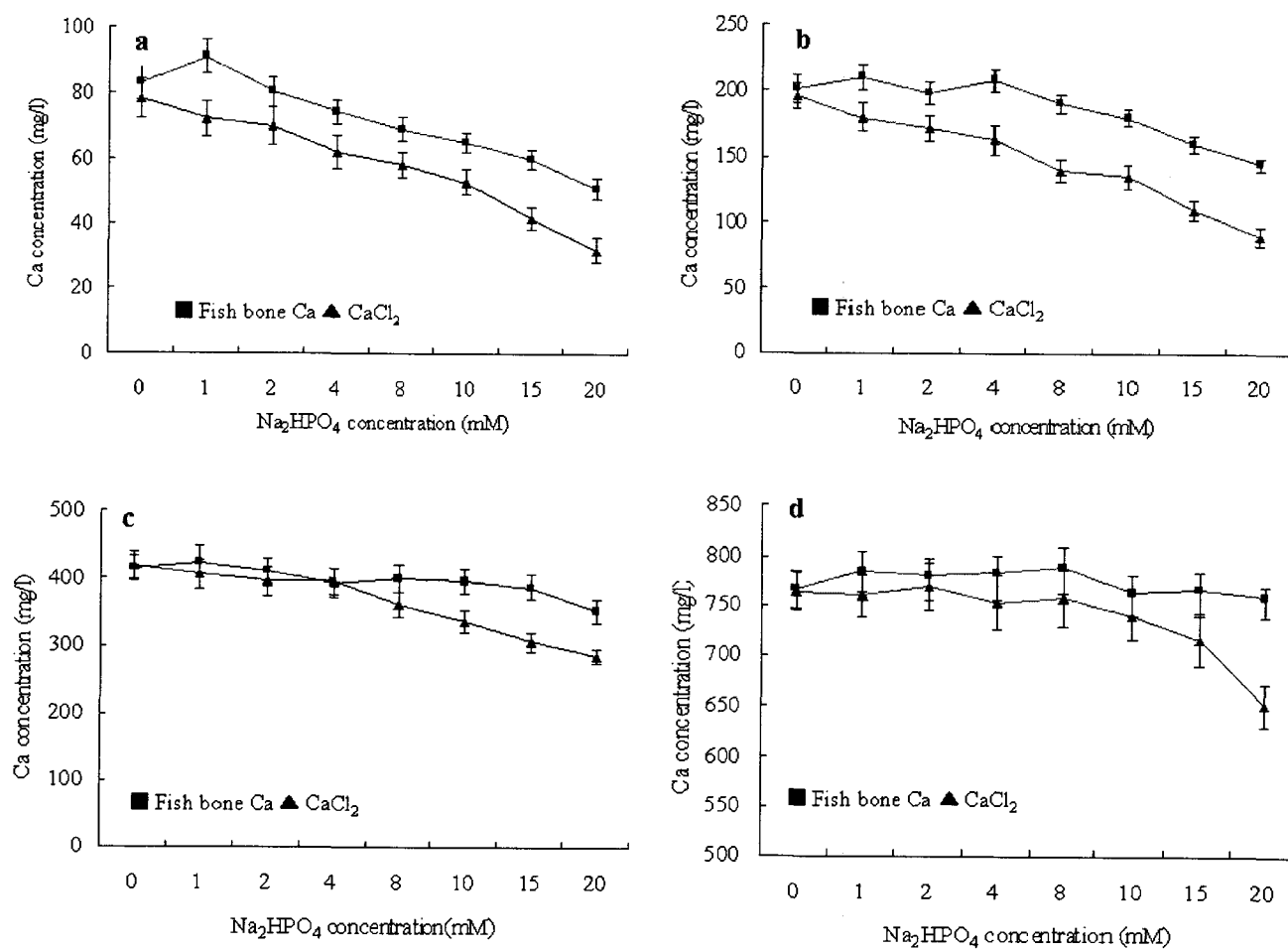

Fig. 3. Binding capacity of fish backbone calcium in presence of Na₂HPO₄. Calcium concentrations were (a) 100 (b) 250 (c) 500 and (d) 1000 mg/L and soluble calcium concentration in the pepsin digest, which was not precipitated, was determined. Fish bone calcium precipitation with phosphate was compared with CaCl₂ solution as a standard at similar conditions. All data were expressed as mean values (mean ± SD, n = 3).

decrease (p<0.05). In contrast, calcium precipitation in the control was increased significantly (p<0.05) greater than that of fish bone calcium, and the fish bone calcium precipitation ($r^2=0.2$) was less correlated with Na_2HPO_4 than $CaCl_2$ ($r^2=0.7$). Further, at 15 and 20 mM Na_2HPO_4 of concentrations fish bone calcium showed significant (p<0.05) greater solubility than that of $CaCl_2$. Thus, it seems that when high calcium concentration and phosphopeptides were present in the alkali media, its solubility was increased, because, at this point, calcium ions bind with phosphopeptides and make soluble complexes (calcium-phosphopeptides), which are the absorbable and bioavailable form to the human body. As reported by Jung et al. (9), 28.76 mg/L of calcium was obtained with hoki bone oligophosphopeptides and solubility of calcium was dependent on fish bone protein concentration. In addition, it was reported that 36.3 mg/L of calcium was obtained with egg yolk phosvitin with 35% phosphate retention (21).

Calcium is one of the difficult minerals to digest and absorb easily, because calcium absorption in the body is controlled by various nutritional and physiological factors. Of total calcium ions, a substantial amount is precipitated with anions like phosphate, carbonate and oxalate under alkali conditions and excrete without being of any use to the body. Therefore, efficient absorption of calcium is decreased by creation of insoluble complexes. Of the calcium phosphate complexes, only calcium dihydrogen phosphate is sufficiently soluble to maintain the essential levels for efficient absorption of the ionic calcium. However, this salt is stable only in highly acidic media. In the alkaline part of the small intestine, the much less soluble mono-hydrogen phosphate of the highly insoluble tertiary phosphate is the stable form, and both of these forms are difficult to absorb completely into the body. To be absorbed properly into the body, calcium should be solubilized under ileum conditions (pH 7.0, 37°C).

Although ionic calcium is present in the small intestine, the bound peptides are not easily precipitated with anions. Therefore, the calcium bound with peptides directly moves to their targets, and is more readily absorbed. Phosphopeptides, especially casein phosphopeptides, amino acids like L-lysine and L-arginine that make calcium soluble within the ileum can stimulate passive diffusion (24-26).

Even though few studies have been done on fish bone protein, many studies are available for milk and egg proteins. Phosphopeptides contained in milk protein can form soluble organophosphate salts and may function as carriers for different minerals, especially calcium (27).

Therefore, these peptides can influence absorption of calcium in the small intestine. In casein phosphopeptides, chelation of calcium with phophoseryl groups plays an important role in enhancing bioavailability of calcium (28). Most casein phosphopeptides contain a serine phosphate cluster and glutamyl residues in the sequence of three phosphoseryl groups followed by two glutamic acid residues. The negatively charged side chains, especially the phosphate groups of these amino acids, define the binding sites for minerals. The significant differences in their calcium binding activity can be attributed to the influence of further amino acids around the phosphorylated binding sites (29). Jiang and Mine (21) found that phosvitin phosphopeptides in egg yolk are effective for enhancing the calcium binding capacity and inhibiting the formation of insoluble calcium phosphate. In phosvitin the serine residues are present as esters of phosphoric acid. Under low ionic strength and acidic conditions, phosvitin becomes water-soluble and can make complexes with calcium ions. Therefore, phosvitin acts as a carrier of calcium (30). In the case of hoki fish bone protein, it was reported that calcium-binding activity was higher than that of casein oligopeptides (9). It was discovered that absorption of calcium from small Bengali fish was comparable to that from skimmed milk (31). Therefore, fish bone calcium could be an important source of calcium for people who consume little or no milk or dairy products. Thus, according to these studies, it can be determined that calcium binds with peptides in the presence of phosphate yielding a soluble bioavailable form of calcium.

Even though some bioactive peptides are not released under physiological conditions *in vivo*, they could be produced commercially and can be used as a supplement or functional food. Casein phosphopeptides are obtained by aggregation of hydrolysate peptides with ultrafiltration (32). Currently, phosphopetide mixtures are commercially available as spray-dried peptide powders. Moreover, it has been proposed that casein phosphopetides could be used for nutritional fortification of foods such as bread, cake, beverages and pharmaceutical preparations (33). Likewise, as a mineral carrier, fish bone peptides might be utilized for improving nutritional content in the food industry, as a better supplement or functional food for calcium deficiency.

In conclusion, we demonstrated feasibility of the separation of bioavailable calcium from Alaska pollack fish backbone by pepsinolytic hydrolysis. The released calcium from pepsinolytic hydrolysis was solubilized effectively in the presence of Na_2HPO_4. This is an effective method to solubilize calcium from fish waste materials

as an under-utilized resource. Further investigation needs to be carried out to improve the technique to use these solublized calciums for human consumption.

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