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Studies on Biological Active Peptide Derived from Fish and Shellfish-Ⅱ Effect of Peptides Derived from Sardine Muscle, Casein and Soybean on Calcium Utilization in Growing Chicks

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The effects of different sources of peptides from enzymatic digestion of proteins and calcium utilization in growing chicks were investigated. The peptides (0.5 g /kg body weight) were orally administered to chicks for 10 days from the 14th day after hatching, and the calcium content of the chick femora were measured. As a result of the measurement of calcium contents / g in the femora after ashing, a significant difference ($p < 0.01$) was confirmed in the high molecular weight peptide fraction derived from sardine muscle (H-SP) and Casein peptide compared with the control group. Further, with respect to the calcium contents per body weight, a significant difference ($p < 0.05$) was also seen in the low molecular weight peptide fraction derived from sardine muscle (L-SP) and Soybean peptide compared with the control group. The peptides, having a molecular weight peak within a range of from 1000 to 5000, showed an Ca-absorption accelerating effect.

1 Introduction

The Utilization of calcium (here-in-after called "Ca") as a component in a living body depends on the degree of Ca-absorption in the intestines rather than the amount of Ca contained in food generally. It has been known for many years that Ca is generally subjected to active transport in the upper part of the small intestine, while it is subjected to passive transport in lower part of the small intestine according to the concentration gradient of Ca. It has also been known for a long time that Vitamin D and Lactose in milk enhance the Ca-absorption. Wasserman *et al.*[1-3] investigated the effect of amino acids on the Ca-absorption in the small intestine. They reported that L-Lisine and L-Alginine both act to promote the Ca-abosorption effectively. Futhermore, Naito *et al.*[4] found that after β-casein and α_{s1}-casein were decomposed by trypsin in the lower part of the small intestine of rats which had been fed cow casein, peptides were generated. It has been proven that those peptides accelerate the transfer of Ca into a living body by preventing the formation of insoluble Ca salts in the intestine. As describes above, various substances relating to Ca-absorption in the intestine are known but the mechanism of action of these substances was not yet elucidated.

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This study describes the effect of peptides derived from sardine muscle, casein and soybean on the Ca-absorption on rapidly growing chicks with excellent Ca-absorption.

2 Materials and Methods

2. 1 Preparation of Peptides Derived from Sardine Muscle, Casein and Soybean

Two point five liter of deionized water was added 500 g of a sardine *Sardinops melanosticta* ordinary muscle, 500 g of casein and 500 g of soybean which had all been boiled beforehand at 95℃ for 2h to homogenize them. The pH of each homogenate was adjusted to pH 2.0 by adding 1N HCl, and 10 g of pepsin (EC 3.4.23.1, Wako Chemicals Ltd.) was added for hydrolysis, and the reaction mixture was kept at 37℃ for 20 h with continual stirring. The hydrolysate was immediately transmitted through an ultrafiltration membrane (YM 10, fraction molecular weight; 10000, Amicon Co.) and the transmitted solution was applied to a strong acidic cation exchange resin, Dowex 50W (H^+form, 50～100 mesh, 4.5×20cm). This column was thoroughly washed with deionized water prior to the elution treatment using 2N NH_4OH, and the eluate was concentrated under a reduced pressure. The concentrated solution was applied to a Sephadex G-25 column (medium, 2.5 × 150cm) preliminarily buffered with deionized water at a flow rate of 30 mℓ /h and fractionated. Each fraction amount was 8.6 mℓ. The peptide fraction was collected by repeating the gel-filtration column chromatography and freeze dried to obtain peptide powder.

The molecular weight of the peptides was determined using Sephadex G-25 column chromatography under the same condition as above. Five hundred mg of the peptide powder, which consisted of insulin (MW=6000), insulin B chain (MW=3500), insulin A chain (MW=2550), bacitracin (MW=1450) and glycine (MW=75) was buffered with a 1M phosphate buffer solution (pH7.0), and then subjected to chromatography. The peptides were detected by the Lowry method.[5)]

Amino acid analyses of the peptides were carried out on the hydrolysates with 6N HCl which contained 0.1% phenol, or 4N methanesulfonic acid which contained 0.2% tryptamine, at 110℃ for 20h using an amino acid analyzer PICO-TAG™ (Waters Ltd.).

2. 2 Animals and Diets

The chicks were housed 5 to a stainless steel cage. The cages were maintained at a temperature of 22 ± 3℃ and humidity of 50 ± 10%. The chicks were fed with commercial feed and given tap water *ad libitum*. The peptide solution was orally administered to the chicks at a volume of 0.5 mℓ (peptide 500mg/kg body weight) per chick once a day from 10 : 00 a.m. to 11 : 00 a.m. everyday. Phisiological saline solution was orally administered to a control group at 0.5 mℓ/ chick. The peptides were orally administered to chicks for 10 days from the 14th day after hatching, and body weight and food intake were measured daily.

2. 3 Measurement of Calcium Content of Femur

After the completion of the administration period, the chicks were sacrificed by bleeding and the right femora were removed. After measuring the wet weight, the removed femora were dried in a hot air dryer at 95℃ for 16 h to measure the dry weight of them. The dried femora were thereafter subjected to 500℃ for 5 h in a furnace in order to ash them and the weight of the ash was measured. The ash was further dissolved in 10 mℓ of 2N HCl and the Ca content of the ash was calculated using an *o*-cresolphthalein complexometric method with a Ca-Measuring set "*O*" (Wako Chemicals Ltd.).

3 Result

3. 1 Preparation of Peptides Derived from Sardine Muscle, Casein and Soybean

The homogenate of the sardine muscle was subjected to autodigestion and pepsin enzymoly-

sis and purified by an ultrafiltration membrane, ion exchange resin column and gel filtration column chromatographies to obtain a low molecular weight of 1000～2000 in the case of pepsin enzymolysis, and a high molecular weight of 2000～3000 in the case of autodigestion (Fig.1). The peptide fractions from a Saphadex G-25 chromatography were pooled and lyophiliyed, the high molecular weight fraction being designated as H-SP, and the low molecular weight fraction as L-SP. The homogenate of casein and soybean was hydrolyzed by pepsin and subjected to the same purifying procedure to obtain a peptide fraction showing a wide molecular weight distribution of 1000～5000 (Figs.2 and 3). The peptide fractions from each Sephadex G-25 chromatography were pooled and lyophilized, the casein peptide being designated as CP, and the soybean peptide as SBP. The results of the analyses of amino acids of the respective peptides are shown in Fig.4. The results show that these peptides had similar amino acid compositions and there was no great difference between them.

3. 2 Food Intake and Body Weight

Peptides were orally administered to chicks for 10 days from the 14th day after hatching. The food intake and gains in body weight of the chicks are shown in Fig.5. The H-SP administered group showed a favorable gain in body

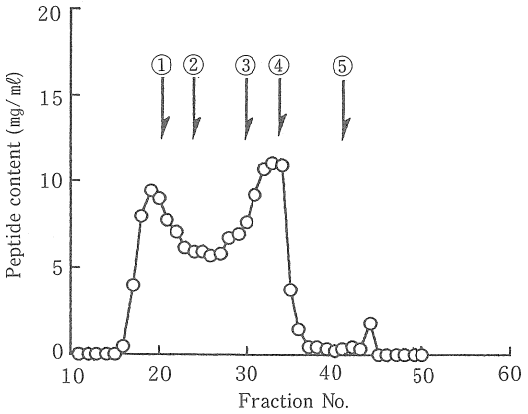

Fig. 2. Gel filtration of Casein peptides on Sephadex G-25. The peptides studied and their molecular weights were the same as legend in Fig.1.

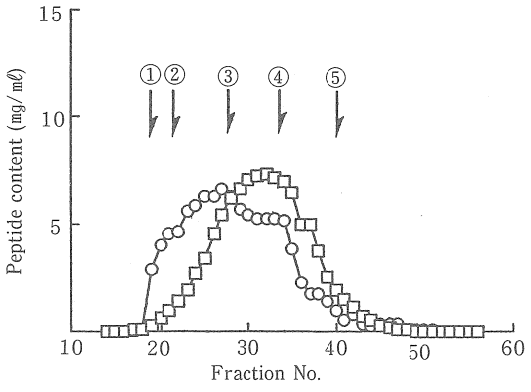

Fig. 1. Gel filtration of Sardine peptides on Sephadex G-25.
○; High molecular weight peptide fraction (H-SP)
□; Low molecular weight peptide fraction (L-SP)
The peptides studied and their molecular weights were ;①Insulin (MW=6000), ②Insulin B chain (MW=3500), ③ Insulin A chain (MW=2250), ④ Bacitracin (MW=1450), ⑤ Glycine (MW=75).

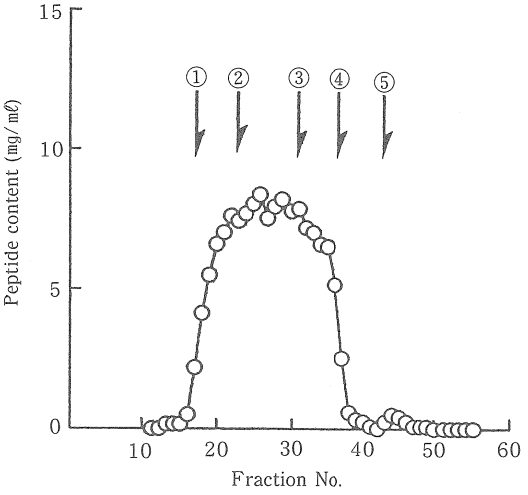

Fig. 3. Gel filtration of Soybean peptides on Sephadex G-25. The peptides studied and their molecular weights were the same as legend in Fig.1.

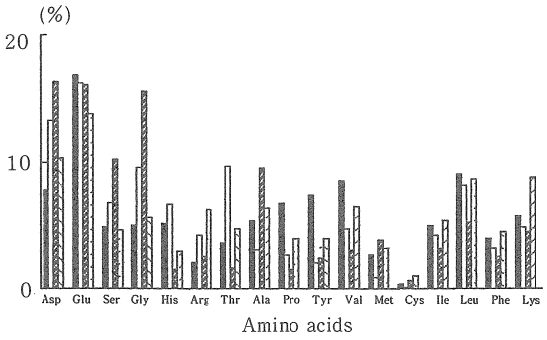

Fig. 4. Ratios of individual amino acid in the peptides derived from sardine muscle, casein and soybean.
■; Sardine peptide (H-SP)
□; Sardine peptide (L-SP)
●; Casein peptide (CP)
●; Soybean peptide (SBP)

weight as compared with the control group. A growth delay was observed in the SBP administered group from the fourth day after administration and this tendency became clearer by the 10th day. Therefore, the SBP administered group had lower food intake and less gain in body weight than the control group and it also exhibited protein-energy malnutrition.

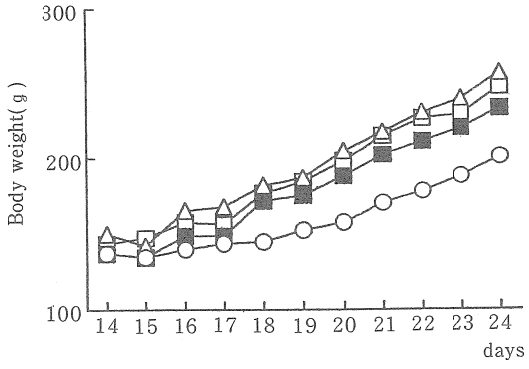

Fig. 5. Changes of body weight in chicks.
□; Sardine peptide (H-SP)
■; Sardine peptide (L-SP)
△; Casein peptide (CP)
○; Soybean peptide (SBP)

3. 3 Calcium Content in Femur of Growing Chicks

After oral administration of the peptides for 10 days, the chicks were sacrificed by feeding. The calcium content in the femora removed from the chicks is shown in Table 1. As a result of the measurement of Ca contents/ g in the femora after ashing, a significant difference ($p < 0.01$) was confirmed in the H-SP and CP administered groups compared with the control group. Further, with respect to the Ca contents per body weight, a significant difference ($p < 0.01$) was seen in the H-SP and CP administered groups compared with the control group. A significant difference ($p < 0.05$) was also seen in the L-SP and SBP administered groups compared with the control group.

4 Discussion

The Ca-absorption in the small intestine is accelerated by Vitamin D. When Vitamin D is taken orally, it is hydrolyzed by hydroxylase of both the liver and the kidney, and becomes active type Vitamin D (1α, $25(OH)_2D$). This substance is carried to the cells of the small intestine in the blood flow and then bonded to the receptors present in the cytoplasm of cells of the small intestine. It is then taken in by the cells, and carried to nuclei to induce the synthesis of protein, such as Ca-bonding protein (CaBP) of low molecular weight considered to be present on the surfaces of microvilli of cells in the upper part of the small intestine (duodenum), taken in by the cells and, thereafter, it is assumed that the Ca is delivered to CaBP in the cytoplasm. CaBP is thought to function as transportation for Ca-ions from the mucous membrane to a srosa which sends the Ca-ions into the blood. As mentioned above, CaBP is believed to play a principal part in the absorption mechanism of Ca in the small intestine and it is known that CaBP contains Lysine as a constitutional amino acid in relatively large quantit-

Table 1. Calcium contents in growing chicks

	Body weight(g)	Wet weight of bone(g)	Dry weight of bone(g)	Ash weight(g)	Ca weight /ash(mg)	Ca weight /body weight(%)
Control	234.2±18.7	1.65±0.23	0.66±0.09	0.25±0.04	82.2±11.8	0.034±0.003
Sardine peptide (H-SP)	248.3±16.6	1.79±0.19	0.71±0.08	0.28±0.03	98.9±10.3[2]	0.040±0.001[2]
Sardine peptide (L-SP)	234.5±16.7	1.65±0.13	0.66±0.05	0.26±0.05	90.6±13.1	0.039±0.006[1]
Casein peptide (CP)	257.1±13.8	1.81±0.19	0.37±0.07	0.28±0.03	97.8± 8.0[2]	0.038±0.003[2]
Soybean peptide (SBP)	201.5±11.9	1.34±0.15	0.54±0.08	0.20±0.024	73.7± 8.9	0.037±0.003[1]

Each value represents the mean±SD.
[1, 2]; $p<0.05$, $p<0.01$ to the control

ies.[6-11]

Wasserman *et al*[1-3] investigated the accumulation of ^{45}Ca in rat femur using various amino acids and rats to which ^{45}Ca was administered, used and reported that there was a marked effect L-Lysine and L-Arginine were used. Dupuis *et al*,[12,13] explained that the absorption accelerating action of Ca from the small intestine due to L-Lysine functioned as follows. Generally, the transport of Ca of the microvilli membrane of the epithelium of the small intestine is obstructed by the phosphorization of membrane protein. If L-Lysine coexists in the actual Ca-absorption, the phosphorization of L-Lysine competes with that of membrane protein participating in Ca-transport centering around the utilization of ATP. At this time, if L-Lysine is present in more than a certain amount, L-Lysine is itself phosphorized to obstruct the phosphorization of membrane protein which in turn enhance Ca-transport. Dupuis *et al.* demonstrated, in experiments using themicrovilli membrane of a rat, that if the concentration of L-Lysine was 10 mM or more, the phosphorization of membrane protein was obstructed. The absorption and utilization of Ca is related to the quantity and quality of protein. The Ca-absorption is slightly enhanced when the dietary protein level is raised but an increase in the discharge of Ca in urine is considered to be mainly caused by a lowering of the re-absorption capacity of Ca in the kidney[14,15] though this mechanism has not yet been proven.

Under this experiment, peptides derived from sardine muscle, casein and soybean were orally administered to chicks, and the effect of peptides on the absorption and utilization of Ca were investigated. With respect to the relation between the molecular weights of peptides and Ca-absorption accelerating effect, the effect is high in the H-SP and CP administered groups. As far as the effect of sardine peptides on Ca-absorption acceleration, it can be said that the peptides with large molecular weights have greater effects. With regard to the relationship between the amino acid compositions of peptides and Ca-absorption accelerating effects, there was no great difference between the amino acid compositions of the respective peptides. Since the preparation of the respective peptides from proteins were not different, it was assumed that the amino acid sequences of peptides influenced Ca-absorption accelerating effects. Futhermore, growth delay was observed in the SBP administered group but there was no effect on the Ca-absorption acceleration. The use of chicks as an *in vivo* screening system is very effective to investigate the Ca-absorption accelaration.

In the future, it would be desiable to digest

peptides derived from food proteins could be studied, to investigate the kinds of peptides useful for aiding Ca-absorption and to find what properties (amino acid structure analysis) obstruct Ca-absorption thereof. It is beneficial to have peptides which control the Ca-absorption accelerating effects present at the time of the processing of food.

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魚介類由来の生理活性ペプチドに関する研究－Ⅱ 発育期ニワトリヒナのカルシウム利用に対するイワシ筋肉，カゼイン，大豆由来ペプチドの影響

末綱邦男・芹澤　功・土井憲豪

各種蛋白質を酵素分解して得られるペプチドを，発育期ニワトリヒナに投与してカルシウムの吸収利用を観察した。ふ化後14日目から10日間，各種ペプチド（0.5g／kg体重）を経口投与した後，ヒナ大腿骨中のCa含量を測定した。灰分当りのCa含量は，イワシ筋肉由来ペプチド（高分子画分）投与群ならびにカゼイン由来ペプチド投与群で有意差（$p<0.01$）をみた。さらに体重当りのCa含量では，これら2画分のみならず，イワシ筋肉由来ペプチド（低分子画分）投与群および大豆由来ペプチド投与群において有意差（$p<0.05$）をみた。これらの結果から，分子量分布1000～5000を持つ各種ペプチドは *in vivo* でのCa吸収促進作用を示した。