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Determination of Amounts of Unfrozen Water in the Solutions of Peptides Derived from Porcine Blood Plasma by Differential Scanning Calorimetry*1

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For the effective use of butchery blood, prior to the usage of the peptides enzymatically derived from porcine blood plasma as a cryoprotectant for fish protein, the unfrozen water and freezing point of peptide solutions derived from various albumins were comparatively measured by a differential scanning calorimetry (DSC). The amount of unfrozen water in the solution of peptide derived from porcine blood plasma (PBP) was 0.39g/g dry matter and 50% solution of peptide derived from PBP showed a freezing point at −12.8℃. The amounts of unfrozen water in the solutions of peptides derived from bovine serum albumin (BSA) and human albumin (HA) were 0.46 and 0.59 g/g dry matter, respectively. Furthermore, 50% solutions of peptides derived from BSA and HA showed a freezing point at −13.0℃ and −12.8℃, respectively.

1 Introduction

Almost all food contains water, and the state of water has great influence on the preservation and quality of the food. In cryorotectants such as sucrose, polyvalent alcohol, and amino acids are used. For example, frozen surimi can be successfully preserved with sucrose. Therefore, it is thought that the hydration of protein plays an important role in the change and freezing denaturation of fish protein. It is known that among amino acids, glutamic acid and asparatic acid have strong cryoprotecting effects. Unfortunately, there have been few studies conducted regarding any direct relationship between the protection effects of cryoprotectant and the behavior of water.[1-5]

One of the methods estimating the states of water in foods is a thermal anaysis. The observation of freezing and thawing process of water by means of the thermal anaysis have been performed for the determination of the amount of unfrozen water by comparing the water content and the peak height in a thawing curve of ice.[6] On the other hand, the freezing curve of foods obtained by the determination with pulse NMR has been proposed.[7,8] Furthermore, a study on the hydration of foods has confirmed the presence of unfrozen water which would be never frozen even at a temperature

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lower than the freezing point.[9-14] In this study, the peptides obtained from a plasma and albumins by enzymatic digestion have been purified, and a method was described for the detemination of the amount of unfrozen water in the peptide solution using a differential scanning calorimetry (DSC). The effects of cryoprotectants and of lowering the freezing point were examined. Some new trials on the relationship detween cryoprotectants and hydration of peptides were also investigated.

2 Materials and Methods

2. 1 Preparation of Peptides Derived from a Plasma and Albumins

Frozen porcine blood plasma (PBP) was provided by the Central Research Institute of Itohamu Food Inc.. Porcine serum albumin (PSA) and bovine serum albumin (BSA) were purchased from Wako Chemicals Co.. Egg albumin (EA), lactalbumin (LA) and human albumin (HA) were purchased from Tokyo Kasei Co..

Five hundred grams of frozen PBP were homogenated in 1*l* of deionized water. Ten grams of pepsin [EC 3. 4. 23. 1] (Merk Co.) were added to the PBP homogenate. The pH of mixture had been adjusted to 2.0 with 1N HCl and the mixture was incubated for 20h at 37℃ with stirring. After the incubation, the reaction mixture was immediately subjected to ultrafiltration through Diaflo-membrane of type YM 10 (ϕ 76mm, Amicon Co.). The filtrate was applied to a column (4.2×15cm) of Dowex 50W×4 (50～100 mesh, H^+ form) cation exchange resin. After being concentrated to 100 m*l* under vacuum, the eluate was applied to a column (2.5×150cm) of Sephadex G-25 equilibrated with deionized water. The eluate was gel-filtrated at flow rate of 30m*l*/h and on fractions of 8.6m*l*. The peptide fraction was collected and concentrated to dryness to give the crude peptide powder (15g).

Fifteen grams of PSA, BSA, EA, LA and HA were homogenated in 100m*l* of deionized water, and one point five grams of pepsin were added to the homogenates of PSA, BSA, EA, LA and HA. The enzymatic hydrolyzation was performed and the peptides were purified by the same methods as described above. The peptide fractions on Sephadex G-25 chromatography were collected and the purified peptide powder (PSA peptide; 4.3g, BSA peptide; 2.8g, EA peptide; 4.7g, LA peptide; 5.2g, HA peptide; 2.4g) were obtained by lyophilization, respectively.

2. 2 Measurment of the Molecular Weight of the Peptide Using Gel Filtration Method [15]

The experiments of molecular weight estimation of peptide were performed with a gel filtration on Sephadex G-25. Two hundred fifty mg of peptide was dissolved in 1m*l* of 0.1M phosphate buffer solution (pH7.0) and the peptide solution was applied to a Sephadex G-25 column (medium, 1.5×150cm) equilibrated with the same buffer solution. The column was eluted with the same buffer solution at a flow rate of 25m*l*/h. Each peptide sample was detected by Lowry method.[16] The peptides used as molecular weight markers are as followed; Insulin (mol. wt. 6,000), Insulin B chain (mol. wt. 3,500), Insulin A chain (mol. wt. 2,550), Bacitracin (mol. wt. 1,450) and Glycine (mol. wt. 75).

2. 3 Measurment of Amino Acid Analysis of Peptide Using HPLC

Amino acid analysis was performed on a PICO-TAG™ amino acid analyzer (Waters Co.). Peptide (10μg) was hydrolyzed with 200μl of 6N HCl including 1% (w/w) phenol in an evacuated sealed glass tube for 24h at 108℃, and the amino acid derivatives were prepared

with phenyl isothiocyanate (PITC). A PICO-TAG™ HPLC system with a reverse phase column (3.9 × 150mm) was used for analyzing the PITC-amino acids. The eluate was chromatographed by linear gradient method using 6% acetonitrile in acetate buffer solution (pH6.4) to 60% acetonitrile for 12 min at a flow rate of 1 m*l*/min. The column temperature was 38℃ and the eluate was monitored by an absobance at 254nm.

2. 4 DSC Analysis [17]

A DSC apparatus (Seiko Instruments Inc., Type DSC-100) was employed. A 20mg of peptide solution adjusted to concentration slightly higher than 5% (w/w) was placed in a silver cell. The scanning was done from −50℃ to 30℃ at a rate of 5℃ min^{-1} with an analytical sensitivity of ± 2 $mcals^{-1}$. The graphical records were drawn as to demonstrate the endothermic changes of the sample at respective concentrations as downward peaks.

When the linear relationship was observed between the water content X (g H_2O / 100g dry matter) and the enthalpy ΔH (cal/g dry matter), the amount of unfrozen water was determined from the slope and intercept of a line. The relationship between the water content X and the enthalpy ΔH was expressed by the equation $\Delta H = AX - B$ (A, B > 0), so the unfrozen water a (g/g dry matter) was calulated by the equation $a = B/(A-B)$.

3 Results

3. 1 Purity and Analysis of Peptides

Fig. 1 shows the molecular weight distribution of the PBP peptide eluted from Sephadex G-25 column. The elution volumes of standard peptides were linear with respect to the logarithm of their molecular weights. A regression line computed from the elution data on the five

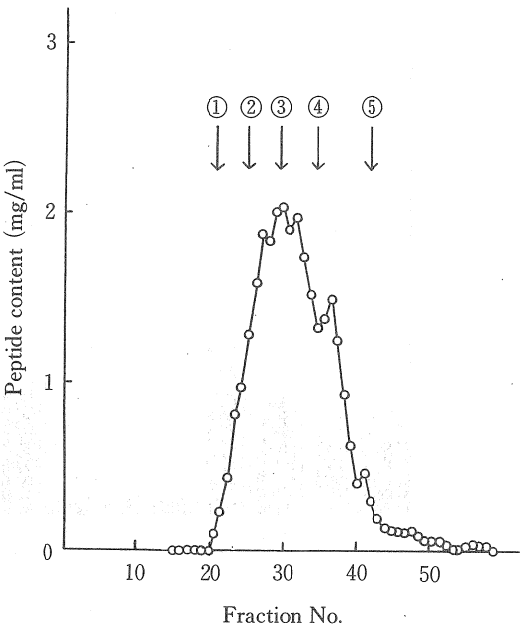

Fig. 1. Column chromatogram of peptide derived from porcine blood plasma (PBP) on Sephadex G-25.
The standard peptides used and their molecular weights were: ① insulin (MW=6,000), ② insulin B chain (MW=3,500), ③ insulin A chain (MW = 2,550), ④ bacitracin (MW = 1,450) ⑤ glycine (MW=75).

of the peptides had a correlation coefficient of − 0.95. The linear range of the column appeared to extend from 300 to 5000. The peptides showed the molecular weight peak within a range of from 1000 to 2000. These results agree with the measurment of molecular weight of peptides using HPLC method.[18]

Fig. 2 shows the ratios of individual amino acids of peptides derived from PBP, PSA, BSA, EA, LA and HA. The levels of the blanched amino acids (Val, Ile, Leu), Ala and Phe were higher than the other amino acids. The levels of Gly and Pro in the BSA and HA peptides were higher than the others. Also the level of Met in the EA peptide, the level of Cys in the LA peptide and level of Lys in the LA and HA

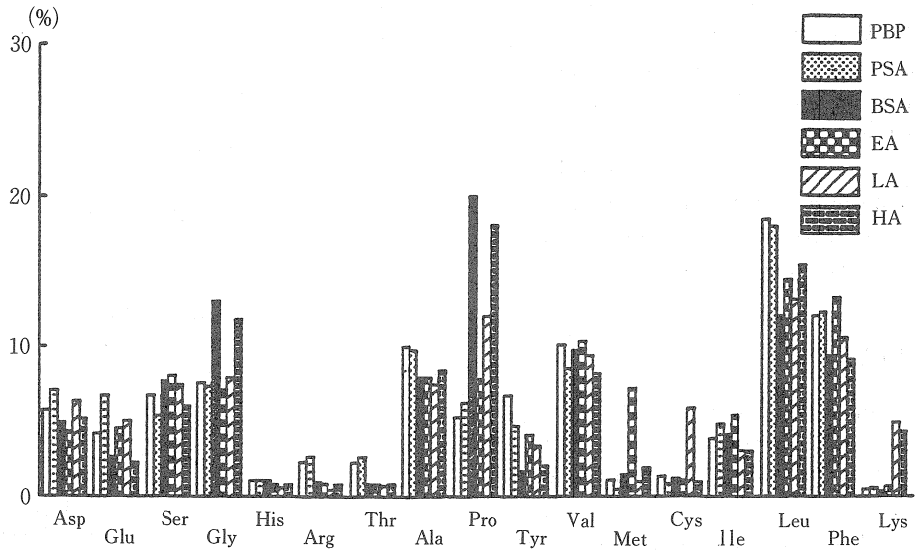

Fig. 2. Aminogram of various peptide fractions eluted from Sephadex G-25.

peptides were higher than the others.

3. 2 Amounts of Unfrozen Water in Peptide Solutions

Fig. 3 shows the DSC thermograms for the various solutions of peptides derived from PBP and the endothermic peaks was observed, respectively.

As shown in Fig. 4, between the water content X of the PBP peptide solution and the enthalpy ΔH accompanied by the thawing, the linear relationships were found.

As shown in Table 1, the amount of unfrozen water in a sucrose solution, which was employed as a standard substance, was 0.463g/g dry matter. The amounts of unfrozen water in the solutions of peptides derived from PBP, PSA, BSA, EA, LA and HA were 0.390, 0.392, 0.465, 0.335, 0.353 and 0.589g/g dry matter, respectively. The amount of unfrozen water in the sucrose solution was 0.46g/g dry matter and 50% sucrose solution showed a freezing point at −7.2℃. The amounts of unfrozen water in the solutions of peptides derived from BSA and HA were 0.46 and 0.59g/g dry matter. Furthermore, 50% of the solutions of peptides derived from BSA and HA showed a freezing point at −13.0℃ and −12.8℃, respectively. The freezing point would be lowered with an increase in the amounts of unfrozen water in each peptide solutions. The higher amounts of unfrozen water in the HA and BSA peptide solutions than the others are related to the amino acid composition. The levels of Gly and Pro in the HA and BSA peptide solutions were characteristically higher than the others.

4 Discussion

The frozen storage of foods is advantageous in that the inherent taste of the food can be maintained as such and that the food can be stored for a long period of time. The denaturation or deterioration of food protein

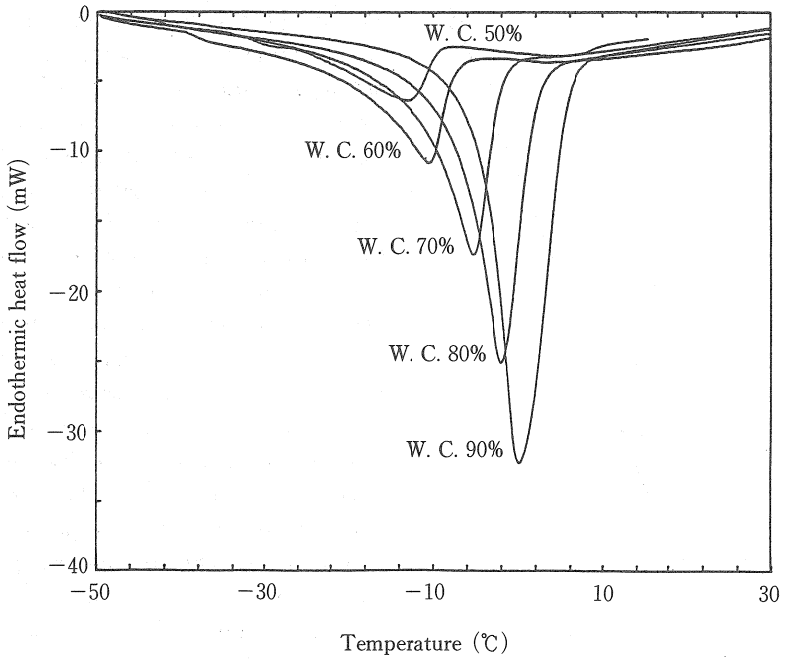

Fig. 3. DSC thermograms of peptide derived from porcine blood plasma (PBP) at different water content.

Table 1. Amount of unfrozen water in peptide solutions and peak temperature of DSC thermograms

Peptide	Unfrozen water (g/g of sold)	DSC peak temp. (℃)				
		W. C.* 90%	80%	70%	60%	50%
Porcine blood plasma (PBP)	0.391	0.13	−2.03	−5.24	−10.43	−12.76
Porcine serum albumin (PSA)	0.392	0.12	−1.16	−5.18	−6.87	−9.72
Bovine serum albumin (BSA)	0.465	−1.30	−2.73	−4.72	−8.11	−12.95
Egg albumin (EA)	0.335	1.20	−0.78	−1.31	−2.20	−4.35
Lactalbumin (LA)	0.353	0.83	−1.67	−3.27	−6.14	−9.54
Human albumin (HA)	0.589	−2.20	−3.82	−5.61	−7.03	−12.76
Sucrose	0.463	1.28	−1.50	−2.74	−4.35	−7.22

*W. C.; Water content (%).

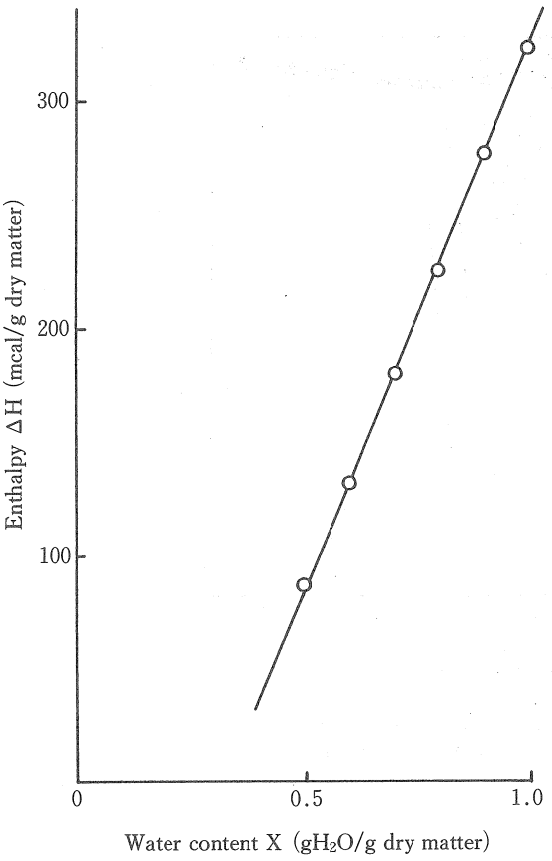

Fig. 4. Relationship between water content and enthalpy in the solution of peptide derived from porcine blood plasma (PBP).

occurred during the freezing. Sucrose and sodium chloride, which are known as the cryoprotectans, can lower the freezing point of the food, however, these solutes largely effect the taste of foods.[19] The amounts of unfrozen water obtained from the DSC analysis in a low temperature, have been quantitatively examined relating to a hydrated water in foods. It is an effect of cryoprotectant on a quality of frozen food and on a change of food ingredient during a frozen storage. A typical example of the prevention of freezing denaturation in food protein with a use of cryoprotectant is a frozen surimi. Althrough large amounts of sucrose, sorbitol and phosphates are used in practice, it is reported that amino acid (glutamic acid) and organic acid (citric acid) are effective cryoprotectants.[20] However, many kind of cryoprotectants might deteriorate inherent taste or odor of foods. It is expected that the peptides and oligosaccharides derived from food protein and food carbohydrate, which have recently attracted special interest as a functional food, may be available for the cryoprotectants.

It is considered that the effects of the cryoprotectants on the dehydrating denaturation of fish protein are attribuable to the physical state of water, namely, the hydration of water molecules with protein. It has been reported to determine the hydration of globular proteins by a pulse NMR technique concerning the unfrozen water in polypeptide solutions.[21-23]

On the other hand, in blood serum from fishes of polar oceans, an antifreeze glycoprotein (AFGP[24]) of repeating units of the tripeptide, Ala-Ala-Thr, lowers the freezing temperatures without affecting the melting temperature. It is instructive to consider that the freezing point would be lowered with an increase in the amount of unfrozen water in the peptide solution. The freezing denaturation of food protein can be prevented by lowering the freezing point and increasing the amount of unfrozen water.

In the present experiment, the PBP, PSA, BSA, EA, LA and HA were prepared by enzymolysis using pepsin and the hydrolysate was further purified by an ultrafiltration membrance, an ion exchange resin column and gel filtration chromatography. In the recent study, we observed the biologically active properties of peptides from enzymatic digestion of PBP and the peptide fractions obtained by these procedures had a peak in a fractional molecular weight of 1,000-2,000.[25]*3,*4 The BSA and HA peptide solution containing large amounts

of unfrozen water, showed lower freezing points around −1.30 to −12.95℃. It is considered that the difference between the amino acid sequence of constitutional peptides exerted the differences in the amount of unfrozen water and in the freezing point. The favorable peptide composition requires clarification in further studies.

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示差走査熱分析によるブタプラズマ由来ペプチド水溶液中の不凍水量の測定

末綱邦男・故越川虎吉

と畜血液の有効利用の一環としてブタプラズマを酵素分解して得られるペプチドを魚肉タンパク質に対する変性抑制物質として利用するための基礎実験に先だち，各種アルブミン由来ペプチド水溶液中の不凍水量と凍結点を測定した。比較のため，牛血清アルブミン，卵アルブミン，ラクトアルブミン，ヒト血清アルブミンについても同様に酵素分解し，得られた分画分子量300～2,000よりなるペプチド水溶液中およびショ糖水溶液中の不凍水量と凍結点を示差走査熱分析を用いて測定した。ブタプラズマ由来ペプチドにおける0.39g不凍水量/g固形物に対し，牛血清アルブミン由来ペプチドは0.46g不凍水量／g固形物，ヒト血清アルブミン由来ペプチドは0.59g不凍水量／g固形物であった。さらに50%ショ糖溶液の凍結点(−7.2℃)に比べ，50%ブタプラズマ由来ペプチド溶液の凍結点(−12.8℃)，50%牛血清アルブミン由来ペプチド溶液の凍結点(−13.0℃)および50%ヒト血清アルブミン由来ペプチド溶液の凍結点(−12.8℃)は低かった。