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Effect of Glucose-6-phosphate Ratio on Functional Properties of Fish Water Soluble Proteins Modified by the Maillard Reaction*

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Fish water soluble proteins (WSP) were modified with different ratios of glucose-6-phosphate (G6P) through the early stage of Maillard reaction, and functional properties of the modified proteins such as pH stability, heat stability, emulsifying activity, water hydration capacity, and protein digestibility were determined. WSP were extracted from blue marlin *Makaira mazara* and mixed with different ratios of G6P (11.7, 23.5, 47, and 94 % of the protein dry weight) prior to be lyophilized. The better improvements of solubility at pHs 7–9, heat stability, water hydration capacity, and protein digestibility were achieved by the modification of WSP with G6P in the ratio of 47 %. Emulsifying activity increased significantly at the early stage of the Maillard reaction with the increasing ratio of G6P. From these results, the modification of WSP with G6P in the ratio of 47 % at the early stage of Maillard reaction was recommended to improve the functional properties of WSP.

Key words: blue marlin, fish water soluble proteins, glucose-6-phosphate, Maillard reaction, functional properties.

Introduction

Population pressure and shortage in proteins of high biological value have encouraged studies on more utilization of by-products in the food processing industries for the production of useful food ingredients. The major portion of the harvested fish is used to produce surimi, a washed and dewatered fish mince, which is further utilized as a raw ingredient for seafood products. The technological development for preparing frozen surimi was responsible for the rapid increase in the production of kamaboko-type products in Japan (Okada *et al.*, 1973). Up to present, the surimi products still occupy the first nomination within fish processing in Japan. About 25 % of the fish catch is processed into kamaboko-type products. Annually, around 800 thousand tons of kamaboko-type products are produced.

One of the most critical steps in the preparation of surimi is washing minced fish flesh with cold water to remove blood, fat, pigments, water soluble proteins (WSP), and other nitrogenous

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compounds. Therefore, the large amounts of fat and WSP of minced flesh are removed by washing and being wasted or unutilized. Nowadays, around 5,000 tons (dry weight) of WSP are annually discarded in the waste water of surimi processing in Japan (Okazaki, 1994). To reduce the cost of waste water treatment and to produce value-added food products, it will be beneficial to recover and utilize these fish proteins.

For successful use as foods, proteins should possess certain critical functional properties. These properties govern suitability of the protein preparation as a food supplement and an ingredient for new food products. Since limited feasibility of using these recovered fish WSP is due to the lack of their functional properties required for food formulation, some modifications have to be carried out in order to attain such functional properties as solubility, emulsification activity, and thermal stability.

Several chemical treatments have been employed in order to improve such functional properties of proteins. The Maillard reaction, one of non-enzymatic methods for modification, has been frequently utilized to improve protein functional properties or some other attributes. The Maillard reaction is known as a constellation of chaotic processes, mediated by a covalent attachment of carbohydrates, lipid, and ε-NH_2 groups of lysine. The deterioration of foods during processing and storage is often caused by the advanced stages of these processes, due to the destruction of essential amino acids, decrease in digestibility, crosslinking with metal ions, along with the formation of antinutritional and toxic compounds impairing consumer acceptability (Friedman, 1996). On the contrary, the controlled Maillard reaction especially in its early stage sometimes gives rise to beneficial phenomena such as improved solubility and heat stability (Kitabake *et al.*, 1985; Wahyuni *et al.*, 1998), production of antimutagens (Yen and Hsieh, 1994), antioxidants (Bedinghaus and Ockerman, 1995), antibiotics (Einarsson *et al.*, 1983), and antiallergens (Oste *et al.*, 1990). The first stable Maillard reaction intermediate is the Amadori rearrangement compound, a ketosyl or aldosylamine N-substituted substance. Subsequent reaction steps include regeneration of the amino groups, deamidation by carbonyl compounds formed from the dehydrated carbohydrate moiety, and formation of carboxymethyllysine (Feather, 1981). These initial steps of the Maillard reaction can thus modify the net charge of proteins by substituting the ε-amino groups. Thus, the Maillard reaction may be a good and safe way of protein modification to improve functional properties, since this phenomenon naturally happens in food systems.

In the previous studies (Wahyuni *et al.*, 1998, 1999), we have succeeded to improve the functional properties of WSP from the flesh of blue marlin through the Maillard reaction with G6P. Phosphorylation of WSP was achieved by using G6P (94 % of the protein dry weight) as a reducing sugar for the Maillard reaction. However, since the use of G6P is costly, it is necessary to reduce the amount of G6P in WSP system for the actual application. The present investigation was undertaken to reveal the suitable ratio of G6P to WSP for improving the functional properties through the Maillard reaction.

Materials and Methods

Modification of WSP with G6P

Blue marlin *Makaira mazara* was obtained as a frozen block from Misaki, Kanagawa Prefecture, Japan. The preparation of fish WSP was described previously (Wahyuni *et al.*, 1998, 1999). Briefly, WSP were extracted from blue marlin flesh by homogenizing with 5 volumes of 0.1 M Tris-HCl buffer (pH 7.6) containing 0.2 mM EDTA. The homogenates were centrifuged at 15,000 xg for 30 min. The supernatants were collected and dialyzed against cold water for 48 h. Proteins in the dialyzate were used as WSP in this study.

G6P with different ratios of G6P such as 11.7, 23.5, 47, and 94 % of the protein dry weight were dissolved in WSP solutions and lyophilized. After the samples were kept in desiccators at 4 ℃ for 4 days to equilibrate relative humidity at 65 % using a saturated $NaNO_3$ solution, they were heated at 60℃ in a water bath for up to 24h for accelerating the Maillard reaction, then kept at-30℃ until use. G6P was purchased from Wako Pure Chemical Ind. Ltd. (Tokyo). All other reagents used were of the highest grade available.

Measurement of Browning and Free Amino Group Content

Forty mg sample was dissolved in 7.5 m*l* of buffer at pH 8.8 containing 2 % SDS, 8 M urea, 2 % mercaptoethanol, and 20 mM Tris-HCl. The development of brown color during the Maillard reaction was determined by measuring the absorbance at 420 nm of the dissolved solutions.

Free amino groups were measured by the 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) method as described by Habeeb (1966). The sample solution (1 mg protein/1 m*l*) was mixed with 4 % sodium bicarbonate and 0.1 % TNBS solution. Prior to the addition of 10 % SDS and 1 N HCl, the mixed solution was incubated at 40℃ for 2h. The absorbance was read at 335 nm. The content of free amino group was expressed as percentage of absorbance per mg protein against unreacted WSP.

Determination of Fructosamine

To evaluate the progress of protein glycosylation, fructosamine content was determined by the method of Johnson *et al.* (1982). The standard used for the determination of fructosamine content was glycosylated human serum (F. Hoffman-La Roche Ltd., Basel). Sample solution (0.1 m*l*) was mixed with 1 m*l* of 0.1 M carbonate buffer (pH 10.8) containing 0.25 mM nitroblue tetrazolium (NBT) at 37℃ and the absorbance at 530 nm was measured after 15 min. Ketamine content (μg/g protein) was used to express the result.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE (5–20% gradient) was performed according to the method of Laemnli (1970).

Forty mg protein was solubilized in 7.5 m*l* of 2 % SDS-8 M urea-2 % mercaptoethanol-20 mM Tris-HCl (pH 8.8) by heating at 100℃ for 2 min. After stirring overnight at room temperature, the dissolved sample was dialyzed against 0.1 % SDS-10 mM Tris-HCl (pH 6.8). Thirty μg protein was subjected to SDS-PAGE. Electrophoresis was performed at 10 mA for 4–5h with 50 mM Tris-0.384 M glycine-0.1 % SDS (pH 8.3) as a running buffer. The protein components were stained with 0.25 % Coomassie brilliant blue R250 and scanned with a dual wavelength flying spot scanning densitometer (Type CS-9300 PC, Shimadzu Co., Kyoto). A molecular weight standard mixture (Sigma Chem., Co., St. Louis, MO) including myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase-b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa) was used.

Solubility-pH Profile

Forty mg sample in 4 m*l* of buffer solution with various pH ranging from 2.0 to 9.0 was homogenized for 1 min and centrifuged at 5,500 xg for 30 min. 0.1 M citric acid buffer (pH 2.0-6.0), 0.1 M maleic acid buffer (pH 7.0), and 0.1 M boric acid buffer (pH 8.0–9.0) were used in this study. The protein concentration of the supernatant was determined using the Biuret method (Gornall *et al.*, 1949). The protein solubility was expressed as percent of protein extracted in the supernatant with respect to that in the unreacted sample. The protein content of unreacted sample was determined by the Kjeldahl method.

Heat Stability

Modified WSP with different ratios of G6P were dissolved at a protein concentration of 5 mg/m*l* in 80 mM Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer to remove unreacted G6P. The dialyzate were diluted to 1 mg protein/m*l* with the same buffer and were heated at 100℃ for 10 min, and immediately cooled in an ice bath. Aggregates were removed by centrifugation at 5,500 xg for 30 min. The protein concentration of the supernatant was measured by the Biuret method (Gornall *et al.*, 1949).

Emulsifying Activity

Emulsifying properties of modified proteins were determined by the spectroturbidimetric method of Pearce and Kinsella (1978). Modified WSP were dissolved at a protein concentration of 5 mg/m*l* in buffer solution and dialyzed against the same buffer for removing unreacted G6P. For the emulsion preparation, 1 g of peanut oil and 3 m*l* of dialyzed WSP (1 mg/m*l*) in buffer solution (pH 7.0) were homogenized by a Hitachi homogenizer HG30 at 18,000 rpm for 1 min at 20℃. Maleic acid buffer (pH 7.0, 0.1 M) was used in this experiment. A 100 μl of sample emulsion was taken from the bottom of the container after different intervals and diluted with 5

m*l* of 0.1 % SDS solution. Absorbance of the diluted emulsion was determined at 500 nm. Absorbance at 0 time was expressed as emulsifying activity of protein.

Water Hydration Capacity

Water hydration capacity of modified WSP was determined by the procedure of Were *et al.* (1997). One gram protein samples were weighed into 30 m*l* centrifuge tubes, then added with 5 m*l* of distilled water. Prior to centrifuging for 10 min at 2,000 xg, the mixture was stirred with a glass rod to form a homogenous paste. The supernatant was carefully removed by decanting and the tubes were weighed to measure the weight of hydrated pellet. Water hydration capacity was calculated by the following equation: water hydration capacity = (weight of hydrated pellet − weight of original sample)/g original sample.

Protein Digestibility

Protein digestibility was measured according the method of Kato *et al*. (1985). Four m*l* of 0.1 % modified WSP solution in 0.05 M Tris-HCl buffer (pH 8.0) was mixed with 250 μ*l* of 0.1 % α-chymotrypsin and incubated at 38℃ for a given time. After protease digestion, 4 m*l* of 4 % TCA solution was added to remove the undigested protein. The precipitate was removed by filtration through a filter paper (Toyo Roshi, No. 5C). The amount of amino acids and peptides in the filtrate was estimated by measuring the absorbance at 280 nm.

Results and Discussion

Development of the Maillard Reaction

The Maillard reaction is a general term used to describe a complex series of reactions between reactive carbonyl groups such as those of reducing sugars and free amino groups such as those found on proteins. Studies on the Maillard reaction of food proteins generally employ indirect methods to provide evidence of Maillard chemistry; these include monitoring the color of browning reaction, loss of free amino groups, and development of fructosamine.

The Maillard browning intensity resulting from heating WSP with different ratios of G6P is presented in Fig. 1. The brown color of modified WSP with ratio of G6P (94 %) developed almost linearly after some induction period of 2h. However, with decreasing ratio of G6P to WSP from 94 %, there appeared significant induction periods for the development of brown color, suggesting that the rate of early Maillard reaction is a function of G6P concentration. It is obvious that the color intensity of all modified fish WSP increased with the reaction time and also with the increase of G6P ratios used.

Figure 2 depicts the change of free amino groups in modified fish WSP with different ratios of G6P used during the modification. The remaining amino groups were expressed as the relative

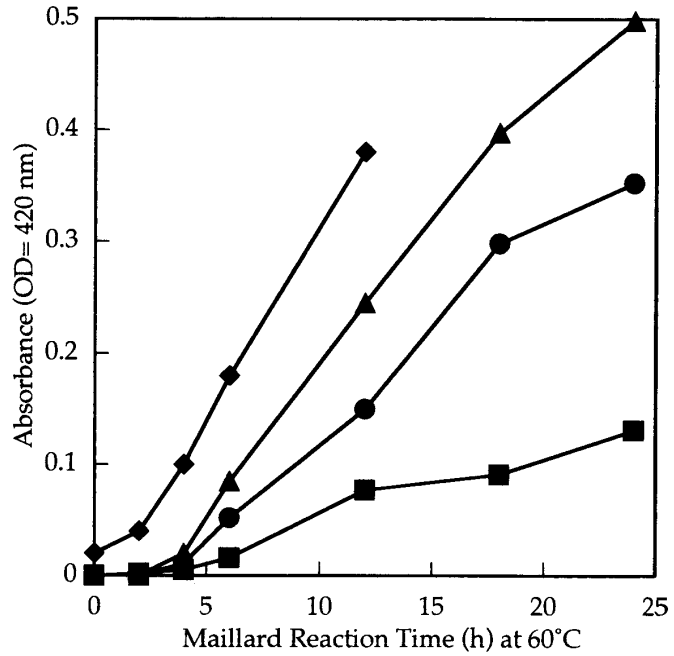

Fig. 1. Effect of G6P ratios to WSP on brown color development.
G6P ratios to WSP were: (■) 11.7 %, (●) 23.5 %, (▲) 47 %, and (◆) 94 %.

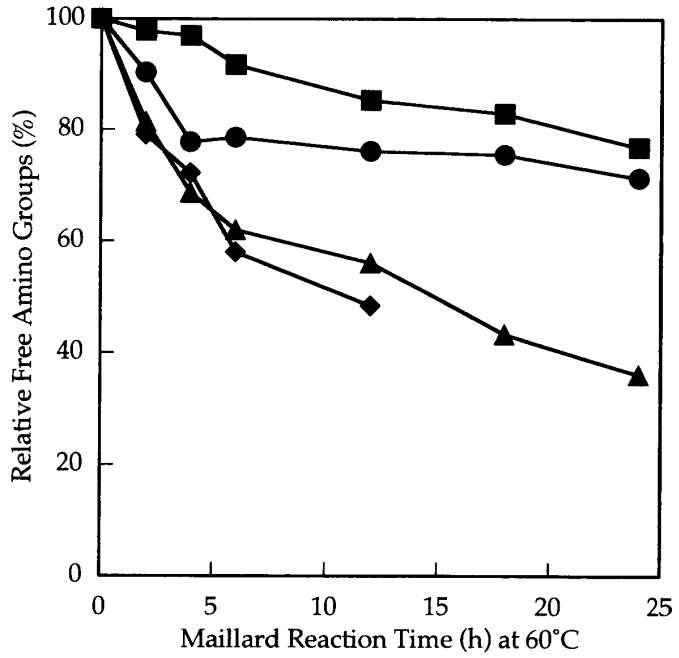

Fig. 2. Changes of free amino groups of WSP during the Maillard reaction.
G6P ratios to WSP were: (■) 11.7 %, (●) 23.5 %, (▲) 47 %, and (◆) 94 %.

value (%) to the free amino groups of unheated WSP. The amount of free amino groups in all modified WSP decreased with the extent of the Maillard reaction time, and the decreasing rates were faster with the increased G6P ratios. Within the first 12h, about 45 and 55 % of the free amino groups of WSP+G6P (1:0.47) and WSP+G6P (1:0.94), respectively, were blocked by

G6P, suggesting that the polar phosphate group of G6P accelerates the advanced steps of the Maillard reaction. In contrast, only 20 % of free amino groups were lost even after 12h of reaction time in other modified WSP with lower ratios of G6P employed.

Nonenzymatic reaction of reducing sugars with protein amino groups initially leads to a rather unstable glycation product or Schiff base which may rearrange to more stable fructosamine or Amadori product. Figure 3 shows the formation of fructosamine during the Maillard reaction. Results are expressed as the ketamine content in this study, since ketamine is formed by the reaction between G6P and amino groups (Wahyuni *et al.*, 1998). Ketamine was formed at the early stage of the Maillard reaction and decreased later in all ratios of G6P employed to modify fish WSP. There was no formation of ketamine observed during heating at 60℃ for modification of WSP with 11.7 % ratio of G6P. The increase of ketamine content was evident with the increase of G6P ratio used in modifying WSP. The subsequent decrease in ketamine content indicated that further progress of the Maillard reaction started after 4h of the reaction in 94 % ratio of G6P to WSP, and 2h of the reaction in lower ratios of G6P (47 and 23.5 %). It is also revealed from Fig. 3 that the early stage of the Maillard reaction was shorter with larger ratios of G6P.

After the modification with G6P through the Maillard reaction, modified WSP samples were subjected to SDS-PAGE with 5–20 % acrylamide gradient gels (Fig. 4). There were obvious changes in electrophoretic protein subunit pattern from 6h of reaction time at the G6P ratios of 11.7 % ad 23.5 % of G6P to WSP. As the reaction period and ratio of G6P increased, the elec-

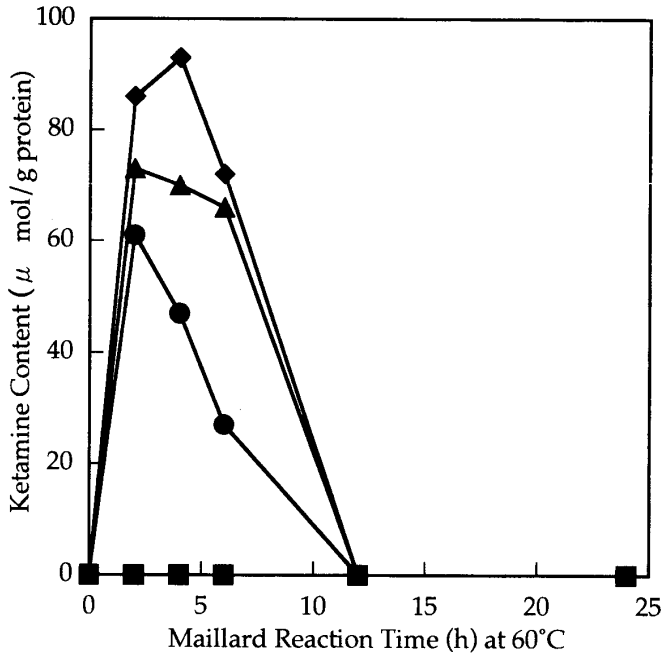

Fig. 3. Formation of fructosamine during the Maillard reaction between WSP and different ratios of G6P. Results are given as the content of ketamine.
G6P ratios to WSP were: (■) 11.7 %, (●) 23.5 %, (▲) 47 %, and (◆) 94 %.

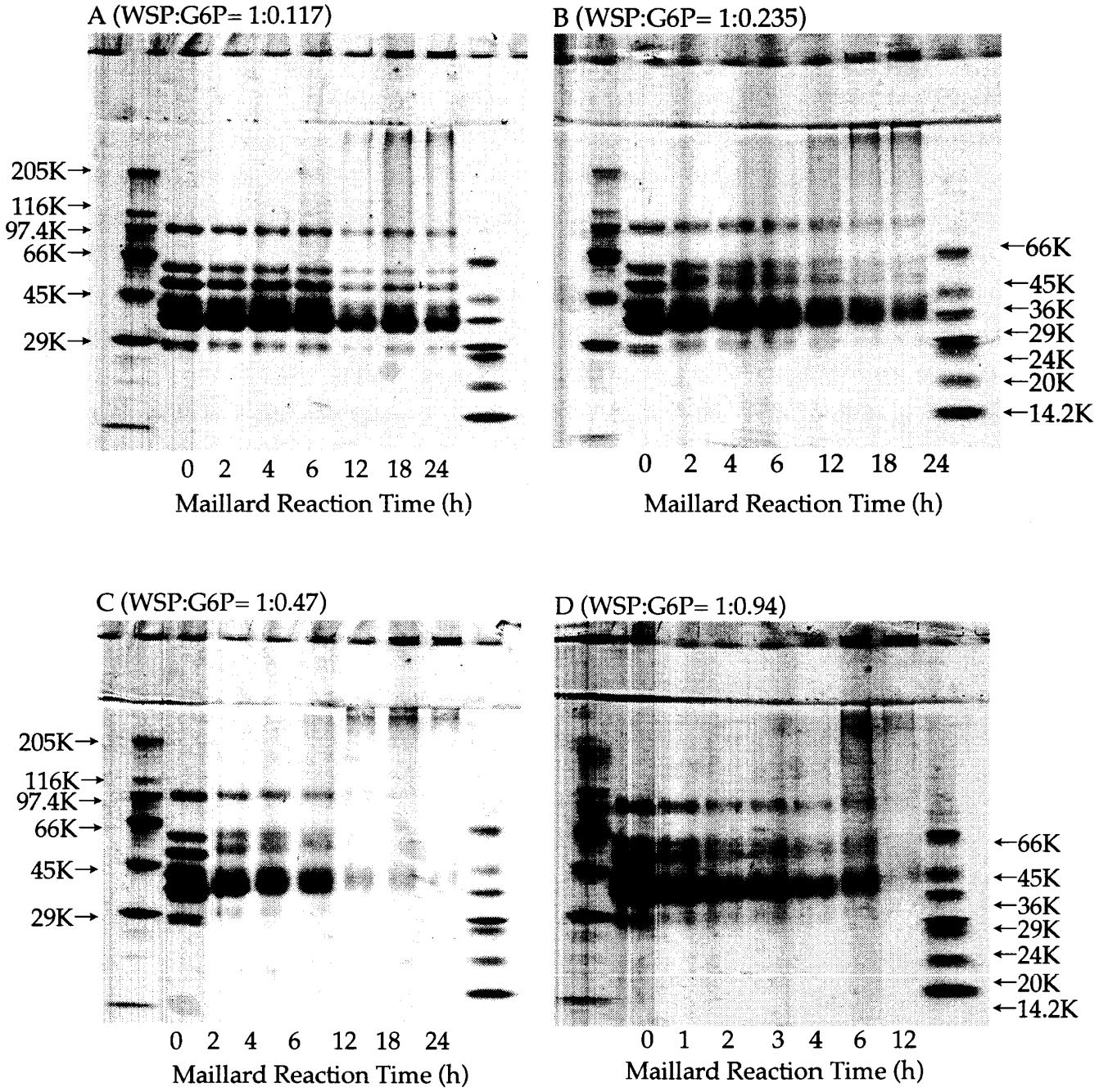

Fig. 4. SDS-PAGE patterns (5–20 % gradient gels) of WSP modified by different ratios of G6P through the Maillard reaction at 60℃.

trophoretic mobility and band intensity of the major protein bands gradually decreased, and protein bands with smaller mobility appeared. Furthermore, the appearance of high molecular weight aggregates which were unable to enter the stacking and resolving gels was observed in WSP reacted with G6P at the later stage of the Maillard reaction, especially with larger ratio of G6P. From the results given by Fig. 4, it is concluded that the Maillard reaction of WSP from blue marlin meat progressed faster with increasing ratio of G6P to WSP. Especially, the development

of the Maillard reaction was significant above G6P ratio of 23.5 % to WSP.

pH-Solubility Profile

Covalent attachment of phosphate groups to protein molecules may be useful in order to change the functional properties of food proteins. Good solubility is desirable to achieve optimal functionality in foods where gelation and emulsification are involved. The solubility profiles of modified WSP at different pH values are illustrated in Fig. 5. It can be seen that there was an increased solubility at neutral and alkaline pH ranges with the increased G6P ratio to WSP. Essentially, similar patterns of protein solubility were observed between modified WSP with 11.7 and 23.5 % ratios of G6P. Phosphorylation of WSP with 47 and 94 % G6P markedly increased the

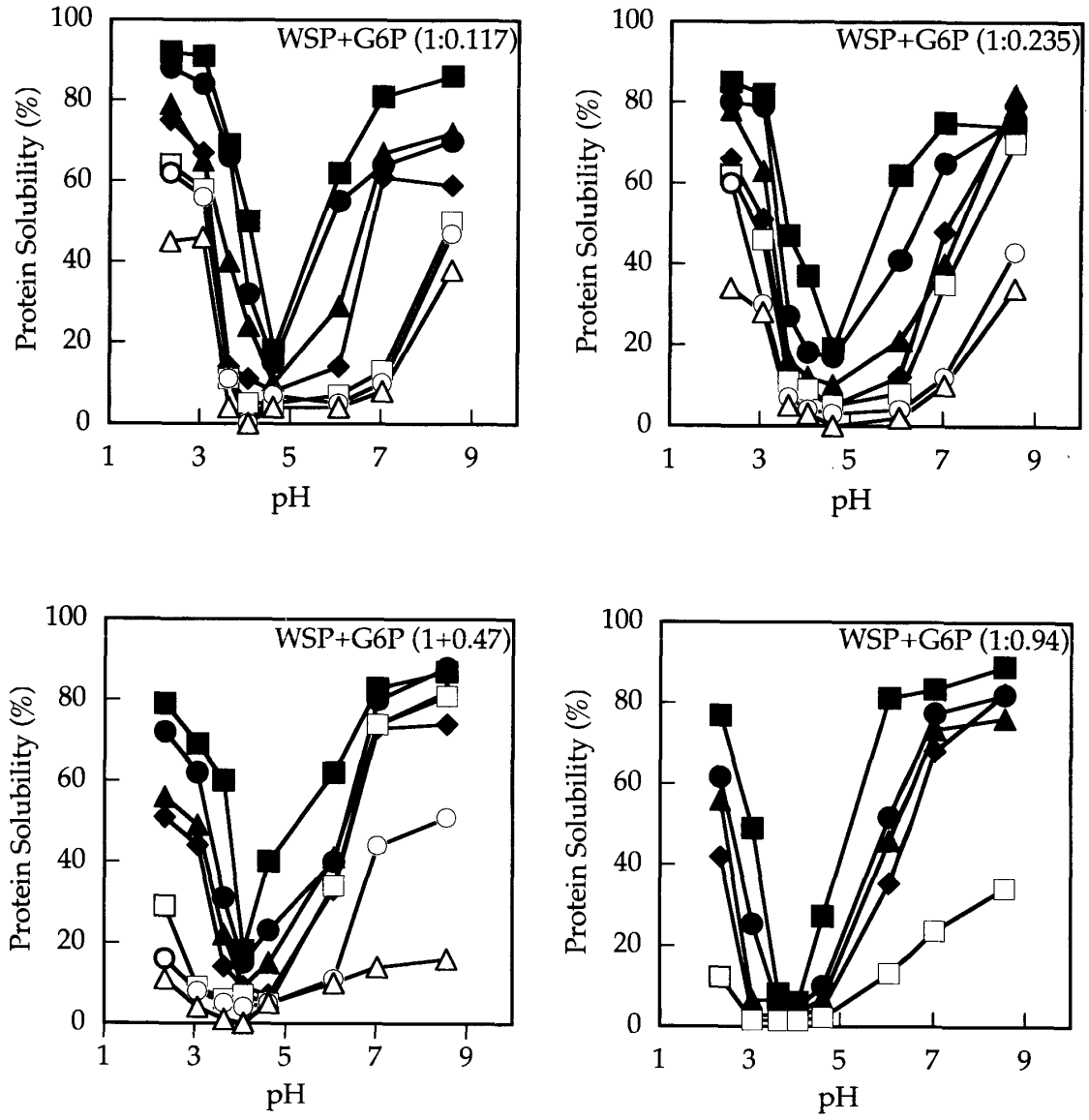

Fig. 5. Solubility profile as a function of pH for WSP modified with different ratios of G6P. WSP were heated at 60℃ for 0h (■), 2h (●), 4h (▲), 6h (◆), 12h (□), 18h (○), and 24h (△).

protein solubility of WSP. Solubility of these modified proteins could be influenced by the changes in the net charge of the protein molecules. The solubility of protein is dependent upon an equilibrium between protein-solvent and protein-protein interactions. Therefore, increase in net negative or net positive charges leads to increased electrostatic repulsive forces between protein molecules, thus favoring protein-solvent interaction. The better protein solubility can be explained by the facts that higher G6P ratio brings about more electrostatic repulsions between protein molecules and produces greater change in the conformation, which results in better protein-solvent interactions.

Heat Stability

The effect of heating temperature on the solubility of modified WSP was determined by heating the samples at 100℃ for 10 min. When unmodified WSP was heated above 60℃ for 10 min, WSP became completely insoluble (Wahyuni *et al.*, 1998; Shimizu and Nishioka, 1974; Kawai *et al.*, 1995). Phosphorylation of WSP with G6P through the early stage of the Maillard reaction significantly improved the heat stability of WSP (Wahyuni *et al.*, 1998). Figure 6 presents the heat stability of modified WSP with different ratios of G6P. When WSP was heated at 60℃ with 11.7 % ratio of G6P, heat stability of WSP was not much improved, but improvement occurred more significantly with the increased ratio of G6P. Heat stability of WSP at 100℃ was gradually improved with increasing Maillard reaction time as well as G6P ratio. At G6P ratio of 47 % to

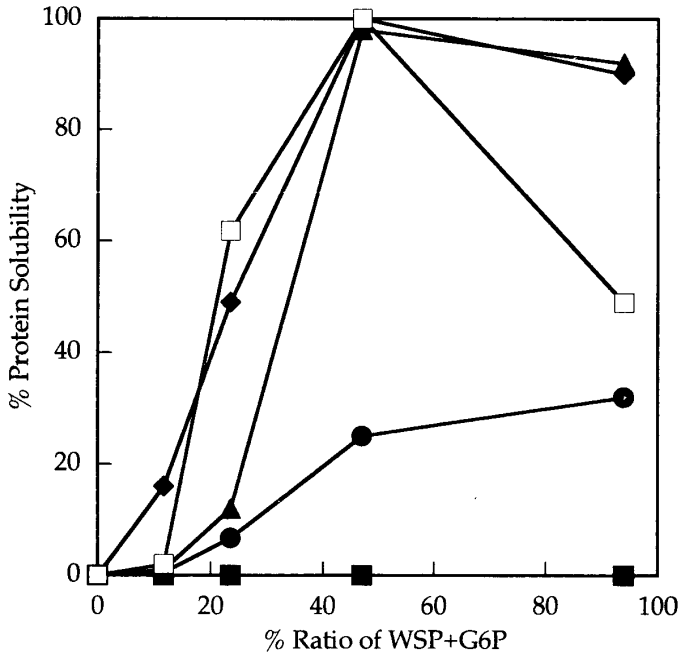

Fig. 6. Effect of G6P ratios to WSP on heat stability at pH 7.0.
WSP were heated at 60℃ with G6P for 0h (■), 2h (●), 4h (▲), 6h (◆), and 12h (□), their solubilities at 100℃ (for 10 min) were determined.

WSP, protein solubility of WSP at 100℃ was almost 100 % after 4h of the Maillard reaction. Then, heat stability was lost to some extent at the advanced stage of Maillard reaction. The increased in heat stability of protein-G6P complex might be due to the electrostatic repulsions among the same negative charges of modified WSP, which protect against precipitation of denatured molecules.

Emulsifying Properties

Emulsifying properties of proteins are important in evaluating their potential use as food additives. Since the ability of proteins to form and stabilize emulsions is critical for many food applications, most of experiments in modification of food proteins have been focused on the emulsifying properties. Figure 7 depicts the changes in emulsifying activity at pH 7.0 of WSP during the Maillard reaction with different ratios of G6P. The emulsifying activity was enhanced with increase in incubation time at 60℃ during the early stage of the Maillard reaction. It is also apparent that emulsifying properties of WSP were significantly improved with the increasing ratios of G6P as well as with the reaction time. This phenomenon seems to be strongly influenced by the hydrophilicity and the amount of covalently bound phosphate groups to WSP through the Maillard reaction, which would take a part in giving the modified protein molecules amphiphilic properties in the emulsion system. The effect of phosphorylation would be promising for the improvement of the emulsifying properties.

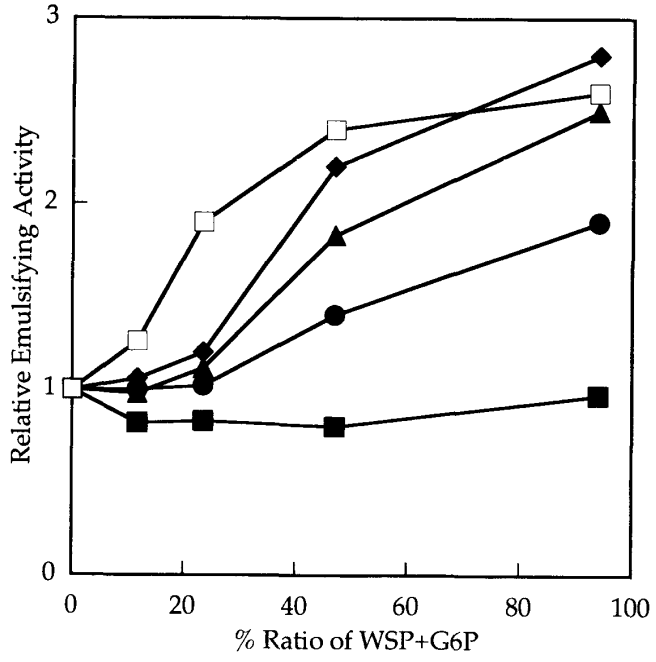

Fig. 7. Effect of G6P ratios to WSP on emulsifying activity at pH 7.0.
WSP were heated at 60℃ with G6P for 0h (■), 2h (●), 4h (▲), 6h (◆), and 12h (□).

Water Hydration Capacity

Apart from influencing solubility, protein-water interactions are also important in determining the nature and extent of water that is bound. This property is commonly referred to water hydration capacity (WHC). WHC is a quantitative indication of the amount of water retained within a protein matrix under a defined condition. Effects of G6P ratios to WSP on WHC of WSP during the Maillard reaction are given in Fig. 8. It is obvious from this Figure that WHC of WSP was remarkably improved by the modification with G6P through the Maillard reaction. It is interesting to note that the improvement of WHC took place in a shorter period when the G6P ratios to WSP were lower, suggesting that WHC increases only at the early stage of the Maillard reaction with G6P. The increase in WHC of modified WSP might be due to both physical and chemical changes of protein structures. Modifications may cause proteins to denature, unfold, and dissociate. Unfolded proteins would have a greater number of water binding sites, which are able to increase WHC. The increased hydration by the added phosphoryl groups and the destruction of protein structure resulted from the repulsion between the negatively charged phosphoryl groups are also engaged in improving the water hydration property.

Protein Digestibility

In vitro digestibility by α-chymotrypsin of WSP during the modification by the Maillard reaction with different ratios of G6P was determined and the results are shown in Fig. 9. The proteolysis rate of glycosylated WSP by α-chymotrypsin considerably increased with the extent ratios

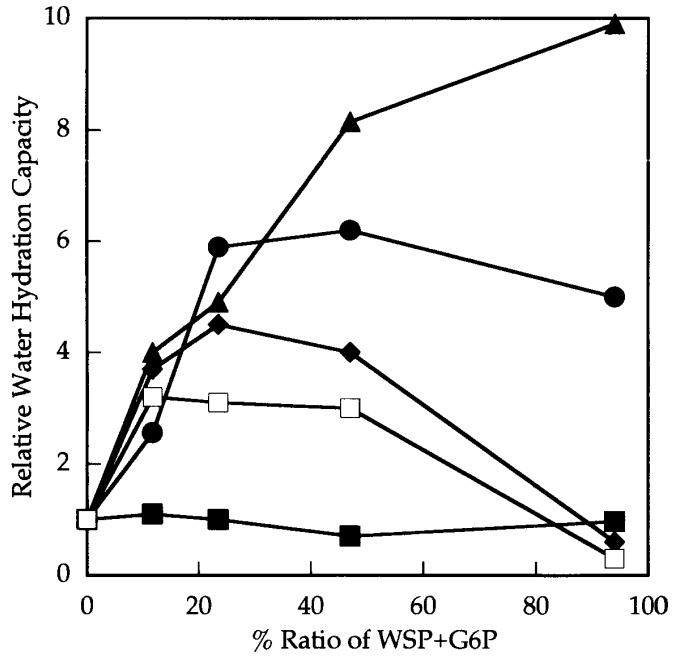

Fig. 8. Effect of G6P ratios to WSP on water hydration capacity.
WSP were heated at 60℃ with G6P for 0h (■), 2h (●), 4h (▲), 6h (◆), and 12h (□).

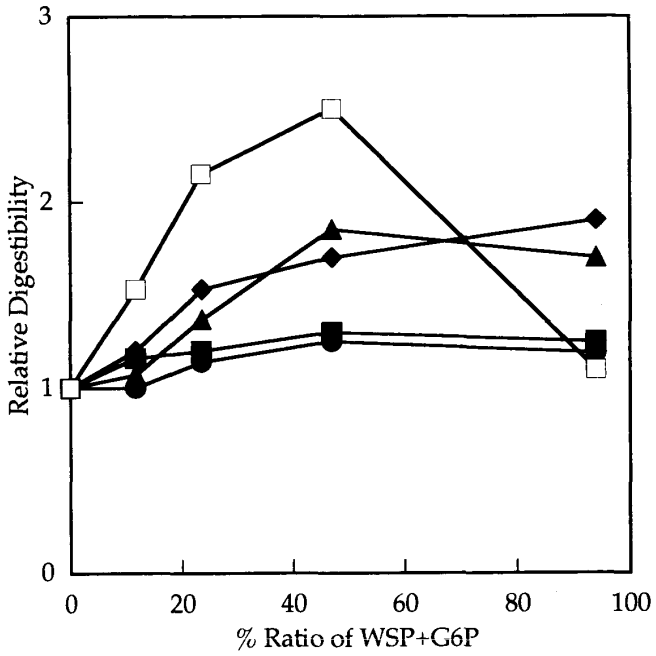

Fig. 9. Effect of G6P ratios to WSP on α-chymotrypsin digestibility.
The Maillard reaction of WSP with G6P was performed at 60℃ for 0h (■), 2h (●), 4h (▲), 6h (◆), and 12h (□).

of G6P up to 47 %, whereas the use of 94 % G6P caused the decrease in digestibility. These findings suggest that the protein structure was markedly affected by the Maillard reaction with G6P. *In vitro* digestibility of phosphorylated proteins has been determined for many proteins (Matheis *et al.*, 1983; Sen *et al.*, 1981) and most of modified proteins had lower digestibility than unmodified proteins. Contradiction of our findings to those results may be owing to the differences in protein and protease sources, phosphorylation procedure, and digestion conditions used.

From these results, it is concluded that the modification of WSP from blue marlin meat with 47 % G6P at the early stage of the Maillard reaction is most suitable for improving the functional properties of WSP. Further study on the effect of phosphorylation through the Mailard reaction on the development of antioxidative activity is now being in progress in our laboratory.

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メイラード反応により修飾した魚肉水溶性タンパク質の機能性に及ぼすグルコース-6-リン酸比率の影響

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各種比率のグルコース-6-リン酸（G6P）を用いて初期段階のメイラード反応によりリン酸化修飾させた魚肉水溶性タンパク質（WSP）のpH安定性，熱安定性，乳化活性，保水性および消化性に及ぼすG6P比率の影響を検討した。水溶性タンパク質はクロカジキ肉から調製し，G6P比率はタンパク質乾重量に対して11.7，23.5，47および94%とした。G6P比率が47%の時，溶解性（pH 7–9），熱安定性，保水性およびタンパク質消化性は最も改善された。WSPの乳化活性はメイラード反応初期段階でG6P比率が大きいほど顕著に増大した。これらの結果より，G6PとのメイラードによるWSPの修飾は，その機能性の改善から判断すると，G6P比率が47%の時に最適であることが明らかとなった。

キーワード：メイラード反応，水溶性タンパク質，G6P，機能性