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Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates

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Abstract

Porcine haemoglobin hydrolysates were prepared through hydrolysis by Alcalase followed by Flavourzyme, and their protein compositions were analyzed using Sephadex G-50 gel filtration chromatography. The antioxidant activities, including reducing power, ferrous ion chelating ability, and DPPH radical scavenging activity, of the hydrolysates were evaluated. The results showed that the hydrolysates of haemoglobin exhibited low reducing powers, but high ferrous ion chelating abilities and DPPH radical scavenging activities. The hydrolysate, obtained through hydrolysis by 2% Alcalase for 4 h and followed by 1% Flavourzyme for 6 h, had the highest ferrous ion chelating ability of 63.54% at a concentration of 5.0 mg/mL. The hydrolysate, obtained through hydrolysis by 2% Alcalase for 4 hrs, had the highest DPPH radical scavenging activity of 41.94% at a concentration of 5.0 mg/mL. According to the results of protein composition analysis, we divided the hydrolysates into three groups, including high molecular weight (MW) group (Group I), medium MW group (Group II), and low MW group (Group III). The reducing power and ferrous ion chelating ability of the hydrolysates were significantly and positively correlated to the relative amount of Group I, and negatively correlated to the relative amount of Group III. This study revealed that the antioxidant activities of porcine haemoglobin hydrolysates were dependent on their protein compositions. The high MW protein fraction (Group I) was responsible for the high reducing power and ferrous ion chelating ability of the hydrolysate.

Keywords: Antioxidant activity; Enzymatic hydrolysis; Porcine haemoglobin; Protein hydrolysate

1. Introduction

Many human diseases are known to be caused by free radicals and natural antioxidants can act as free radical scavengers. Hence, the relationship between human health and the minor nutrients possessing antioxidant activity has always been a popular research subject (Gill & Cross, 2000; Gill, Lopez-Fandino, Jorba, & Vulfson, 1996; Lindmark-Mansson & Akesson, 2000; Meisel, 1997).

Protein hydrolysate is obtained through hydrolysis of protein, and is a mixture of proteoses, peptones, peptides, and free amino acids. Some protein hydrolysates have been found to have specific functional activities (Cempel, Aubry, Piot, & Guillochon, 1995), such as immunological regulatory activity (Cross & Gill, 2000; Mills, Alcocer, & Morgan, 1992), inhibitory activity on angiotensin I converting enzyme (Maruyama, Nakagomi, Tomozuka, & Suzuki, 1985; Hyun & Shin, 2000; Pan, Luo, & Tanokura, 2005), antigenotoxic activity (Park & Hyun, 2002), and antioxidant activity (Carlsen, Rasmussen, Kjeldsen, Westergaard, & Skibsted, 2003; Je, Park, & Kim, 2005; Wu, Lin, Chiang, & Chang, 2004).

Many animal and plant proteins can be used as materials for producing protein hydrolysates. However, from the standpoints of environment protection, a feasible and economic way to produce protein hydrolysates is to use waste materials from farm or food plants. Animal blood produced during slaughtering is a valuable protein source,

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and has been found to produce several bioactive peptides. Some bioactive peptides isolated from the hydrolysate of blood haemoglobin exhibit opioid (Zhao, Garreau, Sannier, & Piot, 1997b), bacterial growth stimulating (Zhao, Coeur, & Piot, 1997a), ACE inhibiting (Hyun & Shin, 2000), and antioxidant activities (Wu et al., 2004). In this study, we used porcine blood to produce protein hydrolysates and investigated the antioxidant properties of porcine blood protein before and after enzymatic hydrolysis.

2. Materials and methods

2.1. Materials

Porcine blood, was obtained from Feng-Lien Co. Ltd. (Pi-Tou Township, Chang-Hua County, Taiwan). The blood collected during slaughtering was immediately mixed with sodium citrate (5 g/l blood), to prevent clotting, and kept below 4 °C until used.

The enzymes used for protein hydrolysis were Alcalase and Flavourzyme (from Sigma Chemicals Co., USA) with declared activities of 2.4 Anson units (AU)/g and 0.5 U/g, respectively.

2.2. Preparation of the haemoglobin sample

The haemoglobin was prepared following a method developed by Nguyen and Chang (1984). The blood was centrifuged at 9970*g* and 4 °C for 10 min to obtain the plasma protein (the supernatant) and the blood cells (the bottom layer). The blood cells were then added with an equal volume of water to burst, and filtered with a piece of gauze to remove the stroma and obtain the haemoglobin. The haemoglobin was freeze-dried and stored at 4 °C until use.

2.3. Quantification of the proximate compositions of the haemoglobin sample

The proximate compositions of the haemoglobin sample, including moisture, crude protein, crude fat, and ash, were assayed using the method of AOAC (1984). Each sample was determined in triplicate.

2.4. Enzymatic hydrolysis

The methods developed by In, Chae, and Oh (2002) and Too and Tsai (1999) for the preparation of hydrolysate were adopted. Five grams of dried haemoglobin were dissolved in 100 ml of 0.1 N NaOH solution at room temperature. The mixture, with a pH of around 6.6, was placed in a water bath maintained at 50 °C, and allowed to stand for 2 h to enable protein denaturation. Reaction pH was adjusted to 8.5 for Alcalase hydrolysis and to 7.5 for Flavourzyme hydrolysis with 4 N NaOH or HCl.

In this study, one- and two-stage hydrolysis processes were conducted. For the one-stage process, Alcalase or Flavourzyme (enzyme:substrate (E/S), 2.0%) was added to the mixture at 50 °C for 0.5, 1, 2, 4, 6, 8, and 10 h. At the end of the hydrolysis period, the mixture was adjusted with HCl to a pH of 4.0 and kept in a 50 °C water bath for 20 min to inactivate Alcalase, or was heated in a 90 °C water bath for 20 min to inactivate Flavourzyme. As for the two-stage hydrolysis process, Alcalase (E/S 2.0%) was used in the first stage and the hydrolysis time was 4 h. In the second stage, Flavourzyme (E/S 0.5%, 1.0%, and 2.0%) was used and the hydrolysis times were 2, 4, and 6 h. Two-stage hydrolysis was stopped by heat treatment at 90 °C for 20 min. The degree of hydrolysis (DH) of haemoglobin hydrolysate was calculated as (amino nitrogen/total nitrogen) × 100%, where the total nitrogen and amino nitrogen content were determined by the semimicro-Kjeldahl method (Hjalmarsson & Akesson, 1983) and formol titration method, respectively.

2.5. Gel filtration chromatography

The proteins of haemoglobin and its hydrolysates were separated on a column of Sephadex G-50 (2.6 × 65 cm). The column was operated in downward flow at room temperature. Phosphate buffer was used to equilibrate the column and to elute the proteins at a flow rate of 48 ml/h. The sample (1.5 ml) at a concentration of 40 mg/ml was applied to the column, and 7-ml fractions were collected. The absorbance of the effluent was measured at 280 nm.

2.6. Preparation of the sample solution for antioxidant properties analysis

Dry haemoglobin and hydrolysate samples were dissolved in distilled water for antioxidant properties analysis. After pre-testing, reducing power and ferrous ion chelating ability were determined at a sample concentration of 5.0 mg/ml, and DPPH radical scavenging activity was measured at a concentration of 2.0 mg/ml.

2.7. Test for reducing power

A method developed by Oyaizu (1986) for testing reducing power was used. The sample solution (10 ml) was spiked with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferric cyanide. The mixture was then kept in a 50 °C water bath for 20 min. The resulting solution was cooled rapidly, spiked with 2.5 ml of 10% trichloroacetic acid, and centrifuged at 800*g* for 10 min. The supernatant (5 ml) was then mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride. The absorbance at 700 nm was measured after reaction for 10 min. A high absorbance was indicative of strong reducing power.

2.8. Test for ferrous ion chelating ability

The method of Decker and Welch (1990) was adopted. Five milliliter of the sample solutions were spiked with

0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine solutions. After reaction for 10 min, the absorbance (at 562 nm) of the resulting solutions was recorded. A complex of Fe^{2+}/ferrozine has a strong absorbance at 562 nm. A high ferrous ion chelating ability in the test sample results in a low absorbance. The ferrous ion chelating ability as a percentage is calculated by [1 − (test sample absorbance/blank sample absorbance)] × 100.

2.9. Test for α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity

A method given by Shimada, Fujikawa, Yahara, and Nakamura (1992) was used to test for DPPH radical scavenging activity. Five milliliter of the sample solution was mixed with 1 ml of freshly prepared 1 mM DPPH methanolic solution. The resulting solution was then left to stand for 30 min, prior to being spectrophotometrically measured at 517 nm. A low absorbance at 517 nm indicates a high DPPH scavenging activity. The DPPH scavenging activity as a percentage is calculated by [1 − (test sample absorbance/blank sample absorbance)] × 100.

2.10. Statistical analysis

In this study, each experiment was conducted in triplicate, and the results were analyzed by one-way analysis of variance and correlation functions in SAS (Statistic Analysis System) (SAS, 1985).

3. Results and discussion

3.1. Proximate composition of the haemoglobin sample

Table 1 shows the proximate composition of the haemoglobin sample. The high ash content might result from the addition of sodium citrate to prevent blood clotting. The results indicate that the haemoglobin sample has a high content of crude protein, and is suitable as a protein source for high-valued functional products.

3.2. Enzymatic hydrolysis of the haemoglobin sample

3.2.1. One-stage hydrolysis

Fig. 1 demonstrates the changes in DH of the hydrolysates during the hydrolysis of haemoglobin by Alcalase and Flavourzyme. It was observed that the DH of the hydrolysates was increased with increasing hydrolysis time. After 1 h, the Flavourzyme hydrolysate exhibited much higher DH than the Alcalase hydrolysate. The DH of Flavourzyme hydrolysate obtained through 10-h hydrolysis was 18.75%, which was 1.91 times higher than that of Alcalase hydrolysate. This is possibly because Alcalase is an endopeptidase, while Flavourzyme is an enzyme complex containing endo- and exopeptidases. An endo- and exopeptidase complex could hydrolyze the peptide bonds in a protein molecule more completely than an endopeptidase. A similar result was also observed by Guerard, Gusimas, and Binet (2002) where the DH of tuna protein hydrolysate obtained through hydrolysis by Alcalase was lower than that obtained by Umamizyme (an endo- and exopeptidase complex).

Table 1
The proximate composition of the haemoglobin sample

Sample	Moisture (%)	Crude protein (%)	Crude fat (%)	Ash (%)
Haemoglobin	0.43 ± 0.02	95.12 ± 0.90	0.75 ± 0.17	2.78 ± 0.09

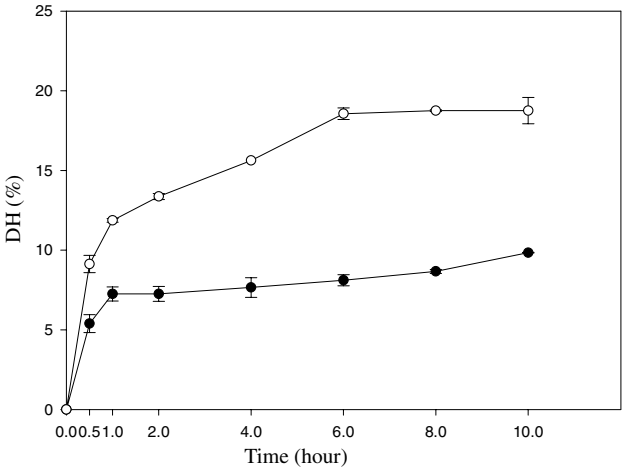

Fig. 1. Changes in DH (degree of hydrolysis) of porcine haemoglobin during hydrolysis by Alcalase and Flavourzyme. Hydrolysis conditions: (-●-) 2.0% Alcalase, 50 °C, pH 8.5; (-○-) 2.0% Flavourzyme, 40 °C, pH 7.5.

3.2.2. Two-stage hydrolysis

Fig. 2 shows the changes in DH of the hydrolysates during the two-stage hydrolysis of porcine haemoglobin by Alcalase followed by Flavourzyme. The DH of the

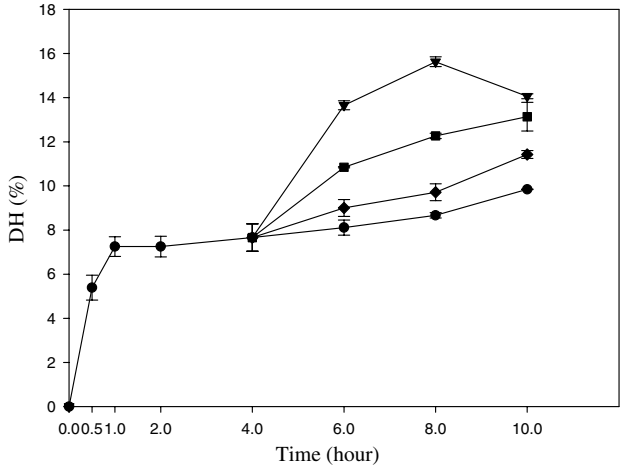

Fig. 2. Changes in DH (degree of hydrolysis) of porcine haemoglobin during two-stage hydrolysis by Alcalase and Flavourzyme. (-●-) First hydrolysis conditions: 50 °C, pH 8.5, 2.0% Alcalase, second hydrolysis on condition: 40 °C, pH 7.5, different concentration of Flavourzyme: (–▼–) 2.0% Flavourzyme; (–■–) 1.0% Flavourzyme; (–◆–) 0.5% Flavourzyme.

hydrolysates obtained through hydrolysis by 2.0% Alcalase for 4 h was 7.66%. When the Alcalase hydrolysate was further hydrolyzed by 0.5%, 1.0% and 2.0% Flavourzyme for 6 h, the DH of the obtained hydrolysates were 11.42%, 13.14%, and 14.06% respectively. Meanwhile, the DH of the hydrolysates obtained through hydrolysis by 0.5% and 1.0% Flavourzyme, increased with increasing hydrolysis time. However, the DH of the hydrolysate obtained through hydrolysis by 2.0% Flavourzyme had a trend of increasing followed by decreasing. The decrease in DH of the hydrolysate obtained through hydrolysis by 2.0% Flavourzyme for more than 4 h was possibly caused by: (1) inhibition of Flavourzyme by the product, or (2) a reverse reaction (peptide formation). It was found that the DH of the hydrolysate obtained through two-stage hydrolysis was higher than that obtained through one-stage hydrolysis. This result is similar to that reported by In et al. (2002).

3.3. Antioxidant activities of haemoglobin and its enzymatic hydrolysates

The antioxidant activities of the haemoglobin and its hydrolysates are presented in Table 2. As observed, at a concentration of 5 mg/ml, the reducing power and ferrous ion chelating ability of the haemoglobin were 0.38% and 74.87%, respectively, which were apparently higher than the values for one-stage hydrolysates of Alcalase and Flavourzyme and two-stage hydrolysates. However, the DPPH radical scavenging activity of the haemoglobin was 21.53%, which was lower than those of one-stage hydrolysates of Alcalase and two-stage hydrolysates but higher than those of one-stage hydrolysates of Flavourzyme. The DPPH radical scavenging activity of one-stage hydrolysates of Flavourzyme was negative in value. It might be caused by haze formation during the addition of the solution of Flavourzyme hydrolysates to the methanolic solution containing DPPH radical.

Table 2
The antioxidant activities of haemoglobin and its enzymatic hydrolysates

Sample	Antioxidant activities[A]		
	Reducing power (Abs. at 700 nm)	Ferrous ion chelating ability (%)	DPPH radical scavenging activity (%)
Haemoglobin	0.38 ± 0.06^{a}	74.87 ± 5.23^{a}	21.53 ± 1.98^{d}
One-stage hydrolysates[B]			
H-A4	$0.21 \pm 0.00^{bc*}$	8.21 ± 3.04^{e}	51.57 ± 4.19^{a}
H-A10	0.17 ± 0.02^{c}	34.50 ± 0.96^{d}	48.55 ± 3.97^{ab}
H-F6	0.08 ± 0.00^{d}	33.96 ± 4.13^{d}	-2.81 ± 0.44^{e}
H-F10	0.10 ± 0.01^{d}	17.94 ± 3.12^{e}	-10.97 ± 3.22^{e}
Two-stage hydrolysates[B]			
H-A4 + F6 (0.5%)	0.24 ± 0.02^{bc}	61.10 ± 0.83^{bc}	38.88 ± 3.44^{c}
H-A4 + F6 (1.0%)	0.23 ± 0.01^{bc}	63.54 ± 3.69^{ab}	41.94 ± 1.89^{bc}
H-A4 + F6 (2.0%)	0.25 ± 0.01^{b}	50.02 ± 2.19^{c}	41.04 ± 1.50^{bc}

[A] The antioxidant activities were determined at a sample concentration of 5.0 mg/ml, except that the DPPH radical scavenging activity was measured at a concentration of 2.0 mg/ml.

[B] Hydrolysis conditions: H-A4, 2.0% Alcalase, 4 h; H-F6, 2.0% Flavourzyme, 6 h; H-A10, 2.0% Alcalase, 10 h; H-F10, 2.0% Flavourzyme, 10 h; H-A4 + F6 (0.5%), 2.0% Alcalase, 4 h + 0.5% Flavourzyme, 6 h; H-A4 + F6 (1.0%), 2.0% Alcalase, 4 h + 1.0% Flavourzyme, 6 h; and H-A4 + F6 (2.0%), 2.0% Alcalase, 4 h + 2.0% Flavourzyme, 6 h.

[*] Values with identical letters in the same column are not significantly different at $p \leq 0.05$.

Comparing the antioxidant activities of one-stage and two-stage hydrolysates, two-stage hydrolysates had higher reducing powers, ferrous ion chelating abilities, and DPPH radical scavenging activities than one-stage hydrolysates, with the exception of lower DPPH radical scavenging activities in two-stage hydrolysates than one-stage hydrolysates of Alcalase. It also could be found in Table 2 that the concentration of the Flavourzyme used in the two-stage hydrolysis had no significant effect on the antioxidant activities, except for ferrous ion chelating ability. The hydrolysates obtained through hydrolysis using 2.0% Alcalase for 4 h followed by 1.0% Flavourzyme for 6 h had higher ferrous ion chelating ability than those hydrolysates obtained from 2.0% Alcalase, followed by 0.5% and 2.0% Flavourzyme.

From Table 2, it could be summarized that a haemoglobin had the highest reducing power and ferrous ion chelating ability; the hydrolysates obtained through one-stage hydrolysis using Alcalase had the highest DPPH radical scavenging activity; and the hydrolysates obtained through two-stage hydrolysis had relatively high reducing power, ferrous ion chelating ability, and DPPH radical scavenging activity. The hydrolysates obtained through one-stage hydrolysis using Flavourzyme had the lowest antioxidant activities, except for ferrous ion chelating ability. This might be due to peptide formation during hydrolysis, resulting in low antioxidant activities, particularly for DPPH radical scavenging activity.

3.4. Sephadex G-50 gel filtration chromatography of haemoglobin and its enzymatic hydrolysates

Fig. 3 shows the Sephadex G-50 gel filtration chromatograms of haemoglobin and its enzymatic hydrolysates. The haemoglobin sample showed a single peak on its chromatogram, meaning that it was not hydrolyzed by enzymes and only contained few low molecular weight molecules. The Alcalase hydrolysates showed two peaks in their chromatograms. The peak containing low molecular weight molecules was much larger than the peak containing high molecular weight molecules. The Flavourzyme hydrolysates showed a complex chromatogram containing one high molecular weight peak and three low molecular weight peaks. Although the profiles of H-F6 and H-F10 had a peak of larger molecular weight peptides, which could be evidence of peptide reverse reaction by Flavourzyme, it also can be seen apparently from this Figure that H-F6 and H-F10 had more peaks of peptides with a molecular weight smaller than H-A4 and H-A10. This result revealed that H-F6 and H-F10 samples were richer in amino groups than H-A4 and H-A10 samples, and hence the DH, calculated based on amino *N*/total *N* ratio, were

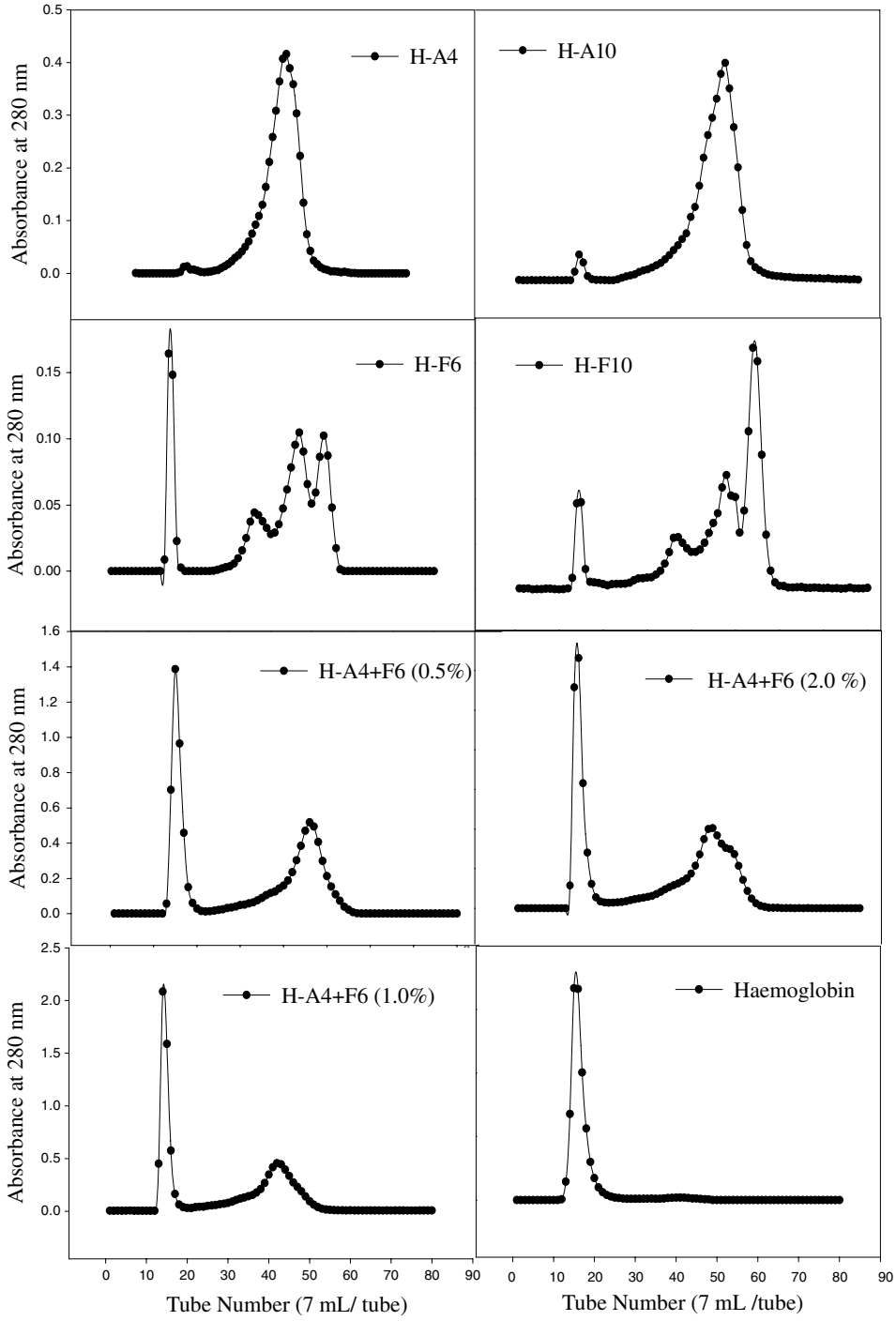

Fig. 3. Sephadex G-50 gel filtration chromatograms of haemoglobin and its enzymatic hydrolysates. Samples H-A4, H-F6, H-A10, H-F10, H-A4 + F6 (0.5%), H-A4 + F6 (1.0%) and H-A4 + F6 (2.0%) are the same as shown in Table 2.

higher for Flavourzyme than Alcalase, which is shown in Fig. 1. The two-stage hydrolysates showed chromatograms similar to those of Alcalase hydrolysates, but with a larger peak of high molecular weight and a smaller peak of low molecular weight than the one-stage hydrolysates of Alcalase.

Based on the results of gel filtration chromatography on Sepadex G-50, we divided the peaks on the chromatograms of haemoglobin hydrolysates into three groups. Groups I, II and III were collected from tube No. 1–20, 21–40 and 41–80, respectively. Table 3 demonstrates the area percentages of these peak groups in the gel filtration chromatograms of porcine haemoglobin and its hydrolysates. It was observed that in the native haemoglobin, the area percentage of Group I was the largest, at 89.31%, and the area percentages of Groups II and III were much smaller, at 7.39% and 3.30%, respectively. In the one-stage hydrolysates of Alcalase and Flavourzyme, the area percentages

Table 3
The area percentages of the peak groups of haemoglobin and its hydrolysates as analyzed by gel filtration chromatography on Sephadex G-50

Sample[A]	Percentage (%)		
	Group I (tube no. 1–20)	Group II (tube no. 21–40)	Group III (tube no. 41–80)
Haemoglobin	89.31[a*]	7.39[d]	3.30[f]
One-stage hydrolysates			
H-A4	1.12[f]	23.89[a]	75.00[b]
H-A10	2.69[f]	12.86[c]	84.46[a]
H-F6	20.31[d]	17.09[bc]	62.60[c]
H-F10	9.83[e]	17.29[bc]	72.88[b]
Two-stage hydrolysates			
H-A4 + F6 (0.5%)	42.38[bc]	13.83[c]	43.79[d]
H-A4 + F6 (1.0%)	47.61[b]	21.53[ab]	30.86[e]
H-A4 + F6 (2.0%)	40.53[c]	15.36[c]	44.11[d]

[A] Samples H-A4, H-A10, H-F6, H-F10, H-A4 + F6 (0.5%), H-A4 + F6 (1.0%) and H-A4 + F6 (2.0%) are the same as shown in Table 2.
[*] Values with identical letters in the same column are not significantly different at $p \leq 0.05$.

of Groups II and III were higher than that of Group I, due to enzymatic hydrolysis.

When one-stage hydrolysates were compared with two-stage hydrolysates, the area percentages of Group III of the Alcalase hydrolysates were the largest, those of Flavourzyme hydrolysates were the next, and those of two-stage hydrolysates were the smallest. It was thought that peptide formation (a reverse reaction of hydrolysis) was occurring during the Flavourzyme hydrolysis, and therefore less low molecular weight hydrolysates were obtained in the Flavourzyme hydrolysates and two-stage hydrolysates.

3.5. Relationships between the protein compositions and the antioxidant activities of haemoglobin hydrolysates

In order to understand the effect of the protein compositions of hydrolysates on their antioxidant activities, we correlated the three peak groups in the gel filtration chromatograms of the haemoglobin hydrolysates to the antioxidant properties of haemoglobin hydrolysates, and the results are shown in Table 4. It was found that the amount of Group I was significantly and positively correlated with the reducing power, while the amount of Group III was significantly and negatively correlated with the reducing power. These results revealed that high molecular weight hydrolysates had higher reducing power than low molecular weight hydrolysates. From the results of ferrous ion chelating ability, it was observed that the amount of Group I was significantly and positively correlated with the ferrous ion chelating ability, and the amount of Group III was significantly and negatively correlated with the ferrous ion chelating ability, which was similar to the results for reducing power. These results revealed that high molecular weight protein hydrolysate (Group I) was responsible for high reducing power and ferrous ion chelating ability of the hydrolysate.

Table 4
The correlation coefficients between the amounts of the three protein groups and the antioxidant activities of the haemoglobin hydrolysates

Protein group	Antioxidant activities		
	Reducing power (Abs. at 700 nm)	Ferrous ion chelating ability (%)	DPPH radical scavenging activity (%)
Group I	0.5428[a]	0.9054[b]	0.1434
Group II	0.0679	−0.3015	0.0924
Group III	−0.5545[b]	−0.8299[b]	−0.1636

[a] Correlation is significant at $p \leq 0.05$ level.
[b] correlation is significant at $p \leq 0.01$ level.

In the results of DPPH radical scavenging activity, the amounts of Groups I, II and III were not significantly correlated with the DPPH radical scavenging activity. However, in Table 2, we found that except for the Flavourzyme hydrolysates, both the Alcalase hydrolysates and two-stage hydrolysates had higher DPPH radical scavenging activities than the native haemoglobin. It revealed that low molecular weight hydrolysates have higher DPPH radical scavenging activities than high molecular weight hydrolysates. The Flavourzyme hydrolysates having low DPPH radical scavenging activities might be attributed to peptide formation during Flavourzyme hydrolysis. Wu, Chen, and Shiau (2003) reported that the DPPH radical scavenging activities of mackerel (*Scomber australasicus*) protein hydrolysates obtained by both autolysis and Protease N increased gradually with increasing hydrolysis time, and the latter was higher than the former. In our research, the DPPH radical scavenging activities of the Alcalase hydrolysates and two-stage hydrolysates were similar to those of the mackerel protein hydrolysates. The fact that the enzymatic hydrolysates possessed DPPH radical scavenging activity may be associated with more low molecular weight molecules. However, the effects of the kinds and the sequences of amino acids of the hydrolysates on their antioxidant activities still need further studies.

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