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Effect of Peroxidized Lipid on the Protein Isolate and Protease Activity of Rice Bran

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Abstract

The destructive effect of peroxidized lipid on the amino acid in protein isolate and the proteolytic activity of protease were studied in the model system of rice bran. The content of amino acids in the protein isolate decreased significantly when they reacted with peroxidized lipid (pov. 1200 meq/kg). Most of amino acids were lost by more than 90% in salt soluble protein isolates when analyzed by the method of enzyme hydrolysis. Formaldehyde reduced the activity most severely among peroxidized products. Formic acid, peroxidized lipid and hydroperoxide were also found to reduce the protease activity. The damaging effect of the secondary products on the protease activity was more serious than that of the primary products of lipid peroxidation. The destruction of amino acids in the total protein and inhibition of protease activity by the peroxidized lipid were apparent.

Key words: peroxidized lipid, protein isolate, protease, damaging effect, model system

Introduction

The peroxidized products of lipid oxidation, such as hydroperoxide and aldehyde can interact with protein, amino acid, enzyme, and biologically active compounds in the biological system. These kind reaction may result in deterioration of quality of food. The lipid-protein interaction can occur through radical reactions[1]. The existence of free radical in α-carbon or side chain carbon in protein and amino acids has been confirmed by Electron Paramagnetic Resonance experiment[2,3].

The possible mechanism of lipid-protein interaction, such as protein-protein cross-links, protein scissions, protein-lipid adducts formation, and destruction of amino acid will occur at reactive site of amino acid[4]. The amino acid residues that are known to be very liable to the damaging effect of peroxidized lipids are -SH, -OH, =NH, and $-SCH_3$[5]. It is much easier to observe the deterioration of quality in protein by measuring the change of enzyme activity than the changes of other functional groups, since even small changes in the microstructure of protein molecule can alter the enzyme activity to a great degree[6]. The reduction of the enzyme activity can be caused either by replacement of the hydrogen in the individual amino acid with the peroxy radical, or by binding of the peroxy radical to the hydrophobic region of the enzyme molecule[7]. Free radical centered in enzyme has been observed when DNA interacts with methyl linoleate[8]. The damaging effect of peroxidized lipid on the protein has been studied intensively in the model system[7-10].

The purpose of this research is to study the destructive effect of peroxidized lipid on amino acid and enzyme. Rice bran protein isolate was prepared as the protein source and protease in the rice bran was partially purified for the enzyme source.

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Materials and Methods

Sample preparation for protein isolates of rice bran

Method of Mitsuda[11] was slightly modified to minimize the damage of protein during the isolation (Fig. 1). Fresh rice bran of the Dong-jin variety, obtained from a local miller located near the Kimhae plain was defatted with n-hexan. Defatted rice bran was extracted with 0.1 M NaCl containing phosphate buffer solution of pH 7.0 for 15 hours in a cold room. Protein isolate obtained from the extraction was fractionated into water and salt soluble protein.

Autoxidation of rice bran oil

Purified rice bran oil, obtained from the Kyongnam Oil Company located near the Kimhae plain, was used. To have the oil autoxidized it was

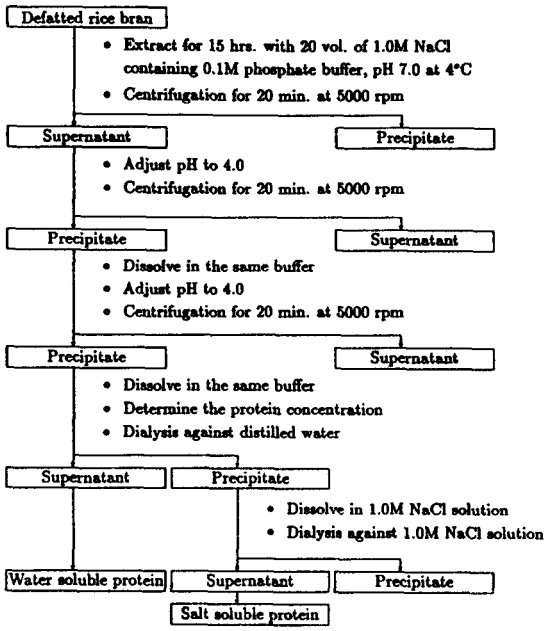

Fig. 1. Schematic diagram for the preparation of rice bran protein isolate

placed in the rotating round flask of the vacuum evaporator and then rotated without vacuum at 50 °C until the peroxide value of rice bran oil reached to 1200 meq/kg.

Solid model system preparation

The peroxidized lipid was emulsified for 3 minutes at 10,000 rpm. To this emulsified lipid, the water and salt soluble protein (4:1/w:w) were added and then mixed thoroughly. Each of the resulting viscous mixture was quickly frozen at -40 °C and then freeze-dried for 48 hours. This freeze-dried sample was stored in a incubator at 50 ± 1 °C for 7 days. To use as a reference protein, water and salt soluble protein isolates, not mixed with the peroxidized lipid, were also freeze-dried.

Amino acid analysis

Total amino acid compositions were analyzed by the method of acid and enzyme hydrolysis. For the enzyme hydrolysis, the method used by Moon[12] was slightly modified in the present experiment. Amino acids in the rice bran were first hydrolyzed with multi enzymes-trypsin (Sigma, 17,000 BAEE units), α-chymotrypsin (Sigma, 51 units/mg solid), peptidase (Sigma, 50 units/g solid), and protease (Sigma, *Streptomyces griseus*. 7.95 mg/ml)-and then the hydrolysates were collected. To the enzyme hydrolysates, sulfsalicilic acid was added to remove the non amino acid materials. Sample was kept at the temperature of 4 °C for an hour and then prepared for the amino acid analysis after it was centrifuged.

Partial purification of rice bran protease

Rice bran was defatted with n-hexan for 15 hours and the meal portion was dried in the air. The defatted rice bran was suspended in 0.01 M phosphate buffer of pH 7.0 and stirred slowly for 15 hours in a cold room. Crude protein extracts were obtained by centrifuging the solution. Crude enzyme was prepared with the protein extracts by ammonium sulfate fractionation.[13] The crude enzyme solution containing 500 mg of protein was applied to the Sephadex G-75 column (3 × 100 cm) which was pre-equilibrated with 0.01 M phosphate buffer of pH 7.0. The protein was eluted with the same buffer. The fractions that showed enzyme activity were collected and then was rechromatographed on Sephadex G-75.

Fractionation of hydroperoxide from rice bran oil

Hydroperoxide in peroxidized rice bran oil (pov: 1200 meq/kg) was fractionated from silica gel column chromatography[14]. The first fraction eluted with 300 m*l* of 2% methanol-benzen solution of autoxidized rice bran oil was discarded and the second fraction eluted with another 200 m*l* of the same solution was collected. The hydroperoxide was confirmed by the thin layer chromatography. The R_f value of the hydroperoxide of rice bran oil obtained from the present experiment was 0.38.

Preparation of aqueous model system

In order to examine the damaging effect of peroxidized products on the proteolytic activity of rice bran protease, rice bran hydroperoxide, peroxidized rice bran oil, formaldehyde, and formic acid were added to the preincubated enzyme solution. Commercially available formaldehyde and formic acid were used as substitutes for the secondary product of peroxidized rice bran oil instead of fractionation. Rice bran hydroperoxide and peroxidized rice bran oil were dissolved in methanol to make a solution of 35% concentration (V/V). And the for-

maldehyde and formic acid was dissolved in distilled water to make 35% (V/V) concentration also. These oxidized products were added to the preincubated enzyme solution to determine the proteolytic activity. Proteolytic activity was determined by the method used by Kim.[13]

Results and Discussion

Loss of amino acids in water soluble fraction of protein isolate by peroxidized rice bran oil

The changes of content of amino acid analyzed by the method of acid hydrolysis are shown in Table 1. Considerable losses in amino acid was observed during the preparation of model system and additional loss was observed during storage at 50 °C for 7 days. Aspartic acid, cystine, glutamic acid, histidine, methionine, phenylalanine, and valine were found to be readily destroyed by the peroxidized lipid. Among these, methionine was damaged most severely. The loss of methionine was 52% during the preparation of model system and 65% during storage at 50 °C for 7 days. Histidine, cysteine, cystine, methionine, lysine, and tyrosine are generally known to be very sensitive to the destructive effects of peroxidized lipid[3-5].

Table 1. Changes in amino acid content of water soluble protein isolate of rice bran by peroxidized lipid g/100g sample (loss, %)

Amino acid	Water soluble protein isolate		
	PI[a]	PI + PL(I)[b]	PI + PL(II)[c]
Alanine	3.08	2.88 (6.9)	2.78 (9.7)
Arginine	3.30	3.00 (9.1)	2.89(12.4)
Aspartic acid	4.68	2.94(25.9)	2.59(26.5)
Cystine	1.60	0.93(42.0)	0.90(43.8)
Glutamic acid	6.91	5.44(21.3)	5.37(22.3)
Glycine	2.70	2.68 (0.7)	2.61 (3.3)
Histidine	1.80	1.32(26.7)	1.27(29.4)
Isoleucine	2.61	2.02(22.6)	1.82(30.3)
Leucine	3.69	2.99(19.0)	2.86(22.5)
Lysine	3.28	2.73(16.8)	2.63(20.0)
Methionine	2.60	1.24(52.3)	0.91(65.0)
Phenylalanine	3.02	1.86(38.4)	1.84(39.1)
Proline	2.23	2.13 (0.7)	1.88 (3.3)
Serine	2.06	2.03 (1.5)	2.00 (2.9)
Threonine	1.77	1.75 (1.1)	1.75 (1.1)
Tyrosine	1.32	1.23 (6.8)	1.03(22.0)
Valine	4.28	2.94(31.3)	2.59(39.5)

[a] PI: Protein isolate.
[b] PI + PL(I): Protein isolate + peroxidized lipid (PI was mixed thoroughly with PL for 2 hours and then freeze dried for 48 hours).
[c] PI + PL(II): Protein isolate + peroxidized lipid (PI + PL (I) was incubated at 50 °C for 7 days).

Loss of amino acids in salt soluble fraction of protein isolate by peroxidized rice bran oil

Total amino acids in salt soluble protein were analyzed by the method of acid hydrolysis and enzyme hydrolysis. Significant losses in amino acid content were observed (Table 2). Most of the amino acids in salt soluble protein isolate are found lost 70-80% of their initial contents when analyzed by acid hydrolysis during the preparation of model system. Additional losses of amino acid during storage was not significant. The loss of methionine, 92% of its initial content, was found to be most significant. The amino acid compositions of enzyme hydrolysates obtained from enzyme hydrolysis were analyzed and compared with the data obtained from the acid hydrolysis. The loss of amino acid was more severe in the enzyme hydrolysis than in the acid hydrolysis. Losses of more than 90% in each of the amino acid were observed in the enzyme hydrolysis. It can be speculated that the amino acid bound to the lipid in the mixture might not have been released by the enzyme hydrolysis but the strong acid might have caused the cleavage of the linkage[10]. Loss of amino acid in the salt soluble protein isolate was found to be greater than in the water soluble. This may be partially attributed to the difference in the conditions of two samples. The reaction mixture of water soluble protein isolate after it was freeze dried was in the state of viscous gel, while salt soluble was in the state of powder. Thus the salt soluble protein isolate probably have more chance to interact with lipid peroxide than the water soluble protein isolate because salt soluble protein have larger surface area.

The severe damaging effect of the peroxidized lipid on amino acid observed in the present study can be explained by the presence of the large amount of peroxidized lipid in the reaction mixture. The amounts of hydroperoxide, aldehyde, and low molecule acid in the peroxidized lipid, whose content was four times that of the protein isolate, were sufficient enough to destroy the amino acid. Matoba[10] found that the damage of amino acid by peroxidized lipid was minimal when the ratio of content of

Table 2. Changes in amino acid content of salt soluble protein isolate of rice bran by peroxidized lipid

g/100g sample (loss, %)

Amino acid	Salt soluble protein isolate				
		Acid hydrolysis		Enz. hydrolysis	
	PI[a]	PI + PL(I)[b]	PI + PL(II)[c]	PI + PL(I)	PI + PL(II)
Alanine	2.00	0.48(76.0)	0.41(79.5)	0.11(94.5)	0.10(95.0)
Arginine	2.54	0.53(79.1)	0.46(81.9)	0.19(92.4)	0.18(92.9)
Aspartic acid	2.88	0.70(75.7)	0.56(80.6)	0.06(97.8)	0.05(98.0)
Cystine	0.84	0.43(48.8)	0.26(69.1)	ND(100.0)	ND(100.0)
Glycine	1.81	0.47(74.0)	0.41(77.4)	0.11(98.4)	0.10(98.7)
Glutamic acid	4.04	0.98(75.7)	0.93(77.0)	0.09(97.7)	0.08(97.9)
Histidine	1.00	0.39(61.0)	0.24(76.0)	0.05(94.9)	0.04(95.1)
Isoleucine	1.88	0.40(78.7)	0.37(80.3)	0.15(92.3)	0.14(92.6)
Leucine	2.80	0.57(79.6)	0.52(81.4)	0.34(87.7)	0.33(88.2)
Lysine	2.09	0.60(77.3)	0.39(81.3)	0.20(90.6)	0.16(92.3)
Methionine	1.30	0.11(91.5)	0.10(92.4)	0.05(96.3)	0.04(97.0)
Phenylalanine	1.78	0.52(70.8)	0.37(79.2)	0.41(77.2)	0.40(77.5)
Proline	1.32	ND(100.0)	ND(100.0)	ND(100.0)	ND(100.0)
Serine	1.17	0.42(64.1)	0.24(79.5)	0.05(95.9)	0.05(95.8)
Threonine	1.19	0.33(72.3)	0.26(78.2)	0.05(95.6)	0.05(96.1)
Tyrosine	0.66	0.23(65.2)	0.20(70.7)	0.12(81.4)	0.12(81.4)
Valine	2.65	0.74(72.1)	0.58(78.1)	0.28(89.7)	0.26(90.2)

[a] PI: Protein isolate

[b] PI + PL(I): Protein isolate + peroxidized lipid (PI was mixed thoroughly with PL for 2 hours and then freeze dried for 48 hours).

[c] PI + PL(II): Protein isolate + peroxidized lipid (PI + PL(I) was incubated at 50 °C for 7 days).

lipid to protein in the reaction mixture was low, but the loss became more eminent as the ratio was increased.

Effect of peroxidized products of lipid on the protease activity

Rice bran hydroperoxide, which is the primary product of oxidized lipid, formaldehyde and formic acid, which is regarded as the secondary products of hydroperoxide, and peroxidized rice bran oil, which contains both the primary and secondary product of oxidation, were used to study the damaging effect of oxidized product on the protease activity. It was found that the oxidized products reduce the protease activity (Table 3). Formaldehyde affected the proteolytic activity most severely. About 60% of the enzyme activity was lost when 50 m*l* of formaldehyde was added. The degree of inhibition of enzyme activity continued to increase as the concentration of formaldehyde in the reaction mixture was raised. Enzyme activity was completely lost when 200 m*l* of formaldehyde was added. Formic acid was also found to affect the protease activity severely. However additional increase of concentra-

Table 3. Effect of peroxidized products of lipid on proteolytic activity of rice bran protease

Products	Amount(μl)	Relative activity(%)
Control	0	100
Hydroperoxide[a]	50	93
	100	87
	200	90
	400	90
Peroxidized lipid[a]	50	66
	100	60
	200	60
	400	60
Formic acid[b]	50	38
	100	36
	200	32
	400	30
Formaldehyde[b]	50	36
	100	20
	200	0
	400	0

[a] The oxidized product of rice bran oil was dissolved in methanol (35%, v:v).

[b] The compound was dissolved in distilled water (35%, v:v).

tion of formic acid did not inhibite the protease activity any further. Hydroperoxide damaged the enzyme activity, but not so significantly. About 7% of enzyme was lost when 50 m*l* of rice bran hydroperoxide was added. And any increase of the damaging effect was not observed as the concentration of hydroperoxide in the reaction mixture was raised. Peroxidized rice bran lipid affected the enzyme activity more severely than the hydroperoxide. It was found that the proteolytic activity is damaged more seriously by formaldehyde and formic acid than by the hydroperoxide and peroxidized lipid. There have been disputes about which one, the primary product of oxidized lipid or the secondary product, has more damaging effect on the protein[4,7,15]. Matshushite[15] reported that proteolytic activity of pepsin was increased by the secondary products, but decreased by linoleic acid hydroperoxide. They also found that proteolytic activity of trypsin decreased by the secondary products, but not affected by linoleic acid.

The reduction of enzyme activity might have been caused either by replacement of the hydrogen in the individual amino acid with the peroxy radical or by binding of the peroxy radical to the hydrophobic region of the enzyme molecule[7]. In fact, free radical centered in enzyme has been observed in the interaction of DNA and methyl linoleate[8].

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미강의 산화 지질이 단백질과 효소의 활성에 미치는 영향에 관한 연구

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미강으로부터 추출한 단백질과 부분 정제한 단백 분해 효소에 미강 지질을 산패시킨 과산화지질 및 이들의 분해산물을 반응시킨 model system에서 아미노산과 효소활성의 변화를 살펴보았다. Protein isolate의 아미노산 조성은 반응 후 현저히 파괴되었으며, 특히 염용성 protein isolate의 경우 90% 이상의 파괴가 관찰되었다. 아미노산 중 aspartic acid, cystine, glutamic acid, histidine, methionine, phynylalanine, 그리고 valine 등은 현저히 감소되었다. Protease 활성의 감소는 formaldehyde와의 반응에서 가장 크게 나타났으며, formic acid, 미강의 과산화지방질, 그리고 미강 산화지질의 hydroperoxide의 순으로 영향을 받았다. 미강 protease 활성 저해에 미치는 영향은 과산화 지방질의 2차 생성물의 영향이 1차 생성물의 영향보다 현저하였다.