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발아 흑미 유래 펩타이드의 개발과 화장품 응용에 대한 연구

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Development of Peptides from the Germinated Black Rice and Applications as Cosmetics Ingredients

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요 약: 발아 과정을 통한 식물 종자의 변화를 확인하고, 발아된 식물종자로부터 새로운 펩타이드를 개발하여 항노화 화장품 소재로서의 가능성을 확인하였다. 발아과정을 통한 식물 종자의 변화는 단백질 양 측정, 겔 투과 크로마토그램(GPC)과 SDS-PAGE를 통한 단백질 분자량 분포 변화, 아미노산 조성분석을 통해 발아 전과 비교하였다. 발아된 검은 쌀로부터 펩타이드를 생산하기 위해 단백질 분해효소를 처리하였으며, 생산된 발아 검은 쌀 펩타이드의 효과를 발아 검은 쌀 단백질과 비교하여 확인하였다. *In vitro* matrix-metalloprotease (MMP) 활성 저해효과는 형광 정량법을 이용하였으며, 인간 섬유아세포 활성화 효과를 확인하였고, 콜라겐 생합성 촉진효과와 자외선 조사에 의한 MMP 발현 저해효과는 효소면역분석법(ELISA)을 이용하여 확인하였다. 발아에 의해 검은 쌀의 단백질량, 단백질 분자량 분포 및 아미노산의 조성이 변화함을 확인하였으며, 검은 쌀의 발아와 단백질 분해효소 중 파파인 처리를 병행함으로서, 상대적으로 낮은 분자량을 갖는 신규 펩타이드의 제조가 가능하였다. GPC를 이용하여 제조된 발아 검은 쌀 펩타이드의 분자량 분포를 확인한 결과, 대부분이 900 dalton 이하의 분자량을 가지는 것을 확인하였으며, 또한, 이러한 발아 검은 쌀 펩타이드는 약 40% 정도의 인간 섬유아세포 활성화 효과를 가지며, 또한 농도 의존적으로 콜라겐 분해효소 활성 저해효과를 가지는 것을 확인하였다. 또한, 자외선 조사에 의해 유도되는 콜라겐 분해효소 발현을 약 50% 억제하며, 콜라겐 생합성을 약 20% 촉진하는 것을 확인할 수 있었다.

Abstract: To develop novel anti-aging peptides from the germinated black rice, we treated with bromelain, papain and Pronase E. And we investigated the effects of the germinated black rice peptide (GBRP) as anti-aging cosmetic ingredients, and compared with the non-germinated black rice protein (NBRP). We investigated the effects on *in vitro* inhibition of matrix-metalloprotease (MMP), proliferation of human skin fibroblasts, stimulation of collagen synthesis and expression of UVA-induced MMPs in human skin fibroblasts. UVA induced MMP-1 expression and collagen contents in human skin fibroblasts were analyzed by enzyme-linked immunosorbent assay (ELISA). As a result, the molecular weight distributions of GBRP and NBRP were determined by gel permeation chromatography to be approximately 900 and 10,000 daltons. GBRP increased skin cell proliferation about 40% and reduced UVA-induced MMP-1 expression about 50%. Also the collagen protein level of cells, which were cultured with GBRP, was increased about 25%. These results suggest that the geminated plant seed peptides can be novel anti-aging ingredients for cosmetics.

Keywords: *germination, rice, protease, peptides, hydrolysates,*

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1. Introduction

Protein active substances for personal care preparations are obtained from natural sources of both animal and vegetable origin, such as wheat, almonds, as well as the soybean and milk protein. Rice is the main grain in Asian countries and is a staple food for many countries. But, most people are familiar with soy and whey protein products. Enzymatic hydrolysis of these plant proteins leads to hydrolysates that posses improved functional properties, as well the potentials as an active substances for personal care preparations. And, germinated plant seeds are receiving increasing attention due to their possible improvement of nutritional qualities, and biological effects. During germination some of the seed storage materials are degraded and used for respiration and partly for synthesis of new cell constituents of the developing embryo, including several enzymes and growth factors. It is well known that a decrease in collagen is shown with photoaging of human skin. Kligman *et al.* reported that a loss of collagen may arise however, from an acceleration of enzymatic degradation due to MMPs release from UV-induced infiltrating cells, in which case the rate of collagen degradation exceeds the rate of biosynthesis[1].

In this paper, a new approach was applied with aimed at to investigate the changes of rice protein during germination; to produce novel peptides from the germinated black rice; to confirm potentials of newly developed peptides as anti-aging agents for cosmetic raw materials.

2. Materials and Methods

2.1. Plant Materials and Enzymes

Seeds of black rice (*Oryza sativa* var. japonica) were soaked in distilled water for 12 h at room temperature (~25℃). The soaked seeds were kept between thick layers of cotton cloth and allowed to germinate in the dark at room temperature for 2 days until the length of the sprout ranged between 5~15 mm. The germinated seeds were rinsed with distilled water, grounded and freeze dried. Pronase E (3.4.24.4) from *Streptomyces griseus* was obtaied from Calbiochem (U.S.A.), Papain (3.4.22.2) and Bromelain (3.4.22.33) were obtaied from Fluka (Switzerland).

2.2. Preparation of the Rice Hydrolysates

The germinated rice flour (10 g) was suspended in deionized water (100 mL) and then heated to 90℃ for 20 min and cool down at room temperature. The acidity of the suspension was adjusted with 2 N HCl and 2N NaOH to the optimal pH according to the enzymes. After the addition of proteases and incubation with shaking for 6 h, the suspensions were heated to 90℃ for 20 min and cool down at room temperature. The enzymatic hydrolysis conditions for papain and bromelain were pH 5.0, a temperature of 45℃, and pH 6.0 and a temperature of 50℃ for Pronase E. The remaining insoluble portion was removed by centrifugation, and the supernatant was lyophilized and stored -20℃.

2.3. Protein and Amino Acids Analysis

Protein content was determined by bicinchroninic acid (BCA) assay. Proteins were analysed by SDS-PAGE (10% polyacrylamide, 0.75 mm thickness) according to the method of Laemmli (1970). Rice proteins were hydrolyzed with 6 N HCl at 110℃ for 24 h and the resulting amino acids were analyzed by AccQ Tag Amino Acid Analysis column (3.9×150 mm, Waters, USA) eluted with linear gradient mode of AccQ Tag eluent A (Waters) and Reagent B (Acetonitrile: water/60 : 40, 0.01%, acetone), at a flow rate of 1 mL/min. The absorbance of eluent was measured at 254 nm. The molecular weight distribution of the rice protein hydrolysates were determined by gel permeation chromatography (GPC) using a Shodex OHpak KB-804 (0.8×30 cm, Showa Denko K.K., Tokyo, Japan) eluted with isocratic mode of 50 mM phosphate buffer, 100 mM NaCl, pH 7.0, at a flow rate of 0.8 mL/min. The absorbance of eluent was measured at 210 nm.

2.4. Cell Treatment and UVA Irradiation

Human dermal fibroblasts (HDFs) were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 10% FBS and kept in a humidified 5% CO_2 atmosphere at 37℃. HDFs from passage 6 to 10 were used in the experiments. Prior to UV irradiation, the cells were washed twice with phosphate buffered saline (PBS). The cells were irradiated from a distance of 15 cm by a UV source (UVA simulator, Jhonsam, KOREA) emit-

ting wavelengths in the range of 340~450 nm. UVA irradiation doses were 5 J/cm^2 and the radiation intensity was measured using UV radiometer (EKO, JAPAN).

2.5. Determination of Collagen and MMP-1 by ELISA

HDFs were seeded into 96-well plates and cultured overnight. After 24 h incubation, the supernatants were transferred into a 96 well plate and the coating buffer (Na_2CO_3 1.59%, $NaHCO_3$ 2.93%, NaN_3 0.20%, $MgCl_2$ 1.02%, pH 9.6) was added 1 : 1 (v/v) and incubated for 12 h. The expression of collagens and MMP-1 was assayed by enzyme-linked immunosorbent assay (ELISA).

3. Results and Discussion

3.1. The Changes of Black Rice Protein by the Germination

The changes of black rice proteins by germination are shown in Figure 1. Proteins were extracted from the various samples through the germination process. During the germination, total soluble protein of black rice was increased by 35% through the germination process (Figure 1(a)). But, the protein contents analysis by SDS-PAGE indicated that the content of low molecular weight proteins was remarkably increased while, the protein contents of high molecular weight proteins were decreased by germination (Figure 1(b)).

3.2. The Changes of Black Rice Amino Acids Compositions by the Germination

The amino acid composition of the black rice protein and the germinated black rice protein was shown in Table 1. During the germination, amino acid composition of black rice seed protein was changed. The proportions of glutamic acid (13.2%) and aspartic acid (8.8%) were increased to 17.3% and 15.4% while, the proportions of glycine and threonine, about 13%, was decreased to 9.0% and 7.3% during germination. The results suggest that, during germination, some of the seed proteins and free amino acids were degraded and used for respiration and partly for synthesis of new cell constituents.

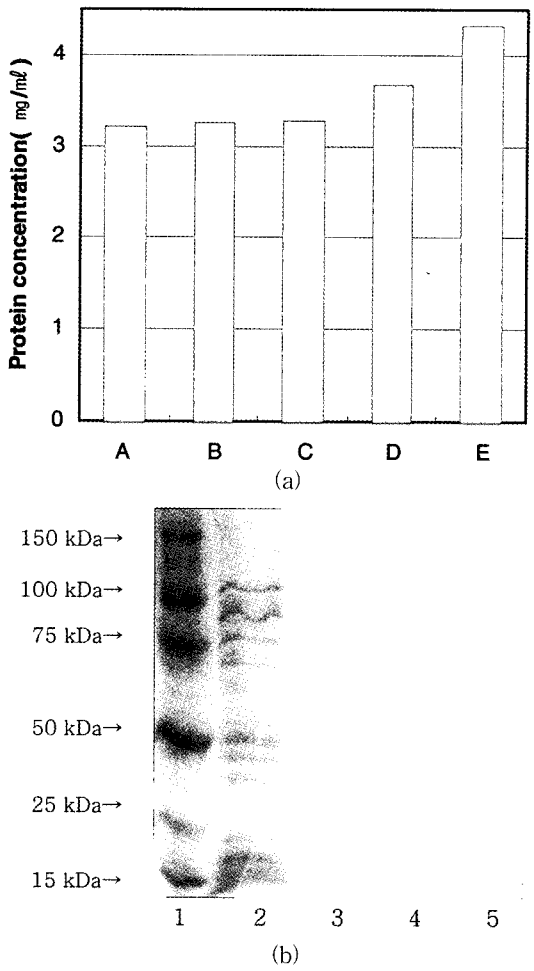

Figure 1. The changes of black rice protein by the germination. (a) Concentration changes of black rice protein by the germination: A. before germination, B. 8 h after the germination, C. 12 h after, D. 24 h after, E. 32 h after, (b) SDS-PAGE of germinated black rice protein.: lane 1. standard marker, lane 2. 8 h after the germination, lane 3. 12 h after, lane 4. 24 h after, lane 5. 32 h after.

Table 1. The Changes of Black Rice Amino Acids Compositions by the Germination

Amino acids	Black rice	Germinated black rice	Amino acids	Black rice	Germinated black rice
Asp	8.8%	15.4%	Cys	1.2%	1.1%
Ser	6.9%	5.6%	Tyr	3.1%	2.4%
Glu	13.2%	17.3%	Val	4.5%	4.7%
Gly	13.0%	9.0%	Met	3.2%	4.0%
His	3.6%	2.3%	Lys	3.6%	5.7%
Arg	4.6%	2.8%	Ile	1.8%	3.1%
Thr	13.2%	7.3%	Leu	2.7%	3.5%
Ala	10.2%	10.1%	Phe	1.4%	1.8%
Pro	4.9%	3.8%			

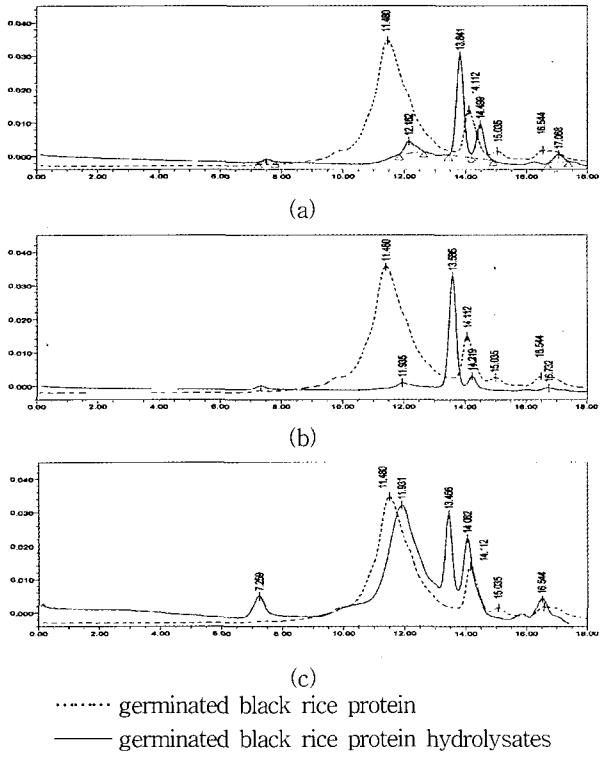

Figure 2. GPC analysis of the black rice hydrolysates. (a) GPC analysis of the germinated black rice hydrolysate by bromelain, (b) GPC analysis of the germinated black rice hydrolysate by papain, (c) GPC analysis of the germinated black rice hydrolysate by pronase.

3.3. Molecular Weight Distribution Analysis of the Black Rice Hydrolysates

Molecular weight distribution of the germinated black rice hydrolysates was analyzed by GPC. The elution profile of the germinated black rice protein showed 2 major peaks (Figure 2). The first, major peak represents a mixture of proteins of high molecular weight. The second contains oligopeptides about 1,000 daltons. After the enzyme hydrolysis, the elution profile of the germinated black rice hydrolysates by bromelain showed 3 major peaks (Figure 2(a)). The first peak (11% of total) represents a mixture of peptides of average molecular weight 3,730. The second, major peak (65% of total) contains oligopeptides about 670 daltons. The final peak of about 360 daltons represents about 24% of the total and corresponds to the tripeptides. And the germinated black rice hydrolysates by papain showed similar elution profile of 3 major peaks (Figure 2(b)). The first peak (2% of total) represents a mixture of peptides of average molecular weight 4,800. The second, major peak (89% of total) contains oligopeptides about 850 daltons. The final peak of about 470 daltons represents about 10% of the total and corresponds to the tetrapeptides. Compared to the elution profile of the germinated black rice proteins, the enzyme treatment effectively hydrolyzed the part of high molecular weight proteins to oligopeptides. Most of the germinated black rice proteins of high molecular weight was dissolved to low molecular weight peptides. But, the germinated black rice hydrolysates by pronase E showed similar elution profile with the germinated black rice protein (Figure 2(c)).

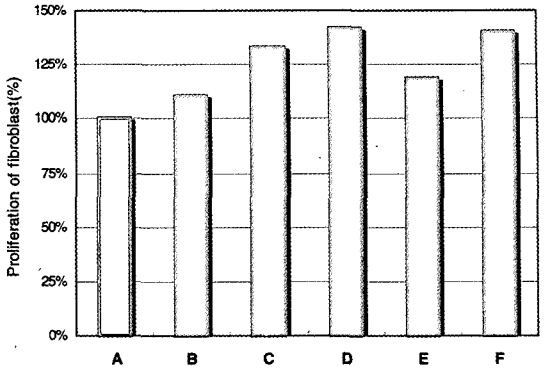

Figure 3. Proliferation of fibroblasts by the germinated seeds hydrolysates. A. control, B. black rice protein 0.2%, C. germinated black rice hydorlysates by bromelain 0.2%, D. germinated black rice hydorlysates by papain 0.2%, E. germinated black rice hydorlysates by pronase 0.2%, F. EGF 10 ng.

3.4. Proliferation of Fibroblasts by the Germinated Seeds Hydrolysates

In the present study, for the germinated black rice hydrolysates, the ability to affect proliferation of skin cells was investigated at a concentration of 0.2% (w/v). First of all, black rice protein showed respectively low effects on proliferation of fibroblasts, it increased cell proliferation about 10% while the germinated black rice hydrolysate by bromelain and papain increased cell proliferation about 33% and 43% (Figure 3).

3.5. Stimulation of Collagen type-1 Synthesis by the Germinated Seeds Hydrolysates

The stimulative effect of the germinated black rice hydrolysates on collagen type-1 synthesis of human

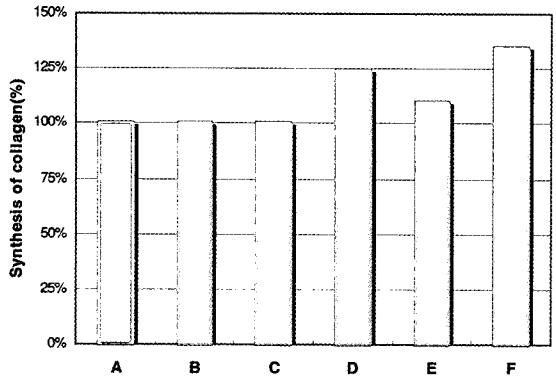

Figure 4. Stimulation of collagen synthesis by the germinated seeds hydrolysates. A. control, B. black rice protein 0.2%, C. germinated black rice hydorlysates by bromelain 0.2%, D. germinated black rice hydorlysates by papain 0.2%, E. germinated black rice hydorlysates by pronase 0.2%, F. vitamin C 200 μM.

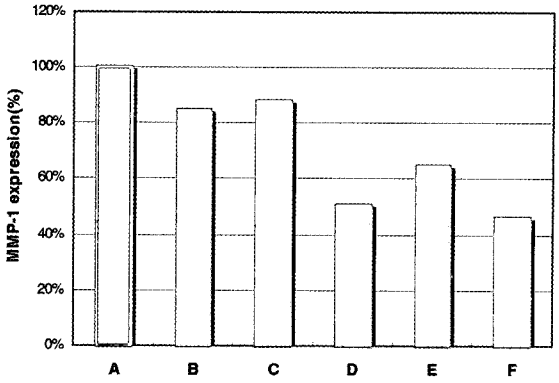

Figure 5. Inhibition of MMP-1 expression by the germinated seeds hydrolysates A. control, B. black rice protein 0.2%, C. germinated black rice hydorlysates by bromelain 0.2%, D. germinated black rice hydorlysates by papain 0.2%, E. germinated black rice hydorlysates by pronase 0.2%, F. vitamin C 200 μM.

fibroblasts was evaluated by ELISA for type I collagen (Figure 4). The black rice protein and the hydrolysate by bromelain showed no stimulative effects on collagen type-1 synthesis of human fibroblasts. While, the collagen protein level of cells, which were cultured with the germinated black rice hydrolysate by papain, was increased about 25%.

3.6. Inhibition of MMP-1 Expression by the Germinated Seeds Hydrolysates

To evaluate effects of the hydrolysates on the MMP production from UVA irradiated HDFs, ELISA were used (Figure 5). To determine whether the hydrolysates could modulate the production of MMP-1 on UVA irradiated HDFs, each sample was applied for 24 h after UVA irradiation to the cells. In the case of black rice protein, it showed respectively low inhibitory effects on UVA-induced MMP expression, about 15%. While the hydrolysate by papain decreased UVA-induced MMP expression about 50%.

4. Conclusion

We could produce novel peptides from the black rice, by combination of the germination and the treatment with protease. The geminated black rice peptides have the effects to activate the skin fibroblast, to stimulate the collagen synthesis and to protect the skin from the damage induced by collagenase. These results suggest that the geminated black rice peptides be novel anti-aging ingredients for cosmetics.

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