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Effect of Low Molecular Weight Silk Fibroin on the Inhibition of Tyrosinase Activity

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Low molecular weight silk fibroin (LMSF), which was prepared by hydrolysis of silk fibroin using high-temperature and high-pressure method, was found to inhibit the oxidation of L-3,4,-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase (EC 1.14.18.1). LMSF contained mostly free amino acids such as L-glycine, L-alanine, and L-serine and oligopeptides, mainly glycine-alanine dimer. As a result of analyzing the inhibition kinetics from Lineweaver-Burk plots, L-glycine and glycine-alanine dimer showed noncompetitive behavior while uncompetitive behavior was observed in L-alanine, and L-serine. When weight percent concentration of ID_{50} was compared, L-glycine was most effective on the inhibition and LMSF was also good enough for the inhibition effect of tyrosinase activity. LMSF showed a mixed-type inhibition and the inhibitory mechanism of LMSF might be caused by free amino acids and oligopeptides. As a result of spectroscopic observation with time, initial rate of increase of DOPAchrome decreased remarkably and the time to reach maximum absorbance increased as an increase of the concentration of L-glycine, meaning that L-glycine made itself mainly responsible for the formation of chelate with Cu^{2+} in tyrosinase. However, in case of L-alanine, L-serine, and especially glycine-alanine dimmer, the production of DOPAchrome after an arrival at maximum absorbance decreased, indicating the production of adducts through the reaction with DOPAquinone.

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Introduction

Mushroom tyrosinase (EC 1.14.18.1) oxidizes L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA) to synthesize melanin (Mayer and Harel, 1979) responsible for browning in fruits and vegetables as well as dermatological disorders, such as melasma, freckles and age spots. In agricultural industry, enzymatic browning is an unfavorable reaction due to a decrease of vitamin C (Synge, 1975) and a retrogression of visual effect resulting in a loss of market values.

To prevent the undesirable reaction, many chemical or natural inhibitors have been reported. The inhibitors are divided into several groups; reducing agents (Embs and Markakis, 1965; Sayavedra-Soto and Montgomery, 1986), chelating agents, compounds reacted with intermediates (Golan-Goldhirsh and Whitaker, 1984), and competitive inhibitors for phenolic substrates. Regardless of strong reducibility of sulfites, alternatives such as L-ascorbic acid (Saper and Ziolkowski, 1987; Hsu *et al.*, 1988) or thiol-containing compounds (Golan-Goldhirsh and Whitaker, 1984), have been sought due to a toxicity of sulfites to body, especially to asthmatics (Taylor and Bush, 1986). Phenolic compounds such as benzoic acid and its derivatives (Robert *et al.*, 1997), 4-substituted benzaldehyde (Jimenez *et al.*, 2001) and arbutin (Maeda and Fukuda, 1996; Sugimoto *et al.*, 2003) belong to competitive inhibitors due to their structural analogues with substrates. Chelating agents are as follows; halide ions, diethyldithiocarbamate, thiourea, cyanide, azide, and EDTA (Duckworth and Coleman, 1970; Healey and Strothkamp, 1981).

Amino acids and peptides can inhibit the tyrosinase

activity by means of more complicated mechanisms including a reaction with intermediates, quinines (Synge, 1975), and a formation of stable Cu^{2+} chelates (Eichhorn, 1973; Viola *et al.*, 1979). Among them, thiol-containing amino acids, L-cysteine, is most effective on the inhibition of tyrosinase activity since a thiol group is easily reacted with quinines (Richard-Forget *et al.*, 1992), has high affinities for Cu^{2+} (Kahn, 1985), and reduces intermediates to substrates (Cilliers and Singleton, 1990).

In our previous study (Kang *et al.*, 2002), low molecular weight silk fibroin (LMSF), which consisted of amino acids and oligopeptides mainly, could be prepared by high-temperature and high-pressure method. Among useful functionalities of LMSF, it is expected that LMSF may be a good inhibitor of tyrosinase due to its components included. Therefore, the purpose of this study is to determine the effect of the inhibition of tyrosinase activity focusing on the components of LMSF, such as amino acids and oligopeptides.

Materials and Methods

Materials

Mushroom tyrosinase (250,000 units/mg), L-3,4-dihydroxyphenylalanine (L-DOPA), L-glycine, L-alanine, L-cysteine, glycylalanine, and L-ascorbic acid were purchased from Sigma (USA). LMSF was prepared by high-temperature and high-pressure method (Kang *et al.*, 2002) at the conditions of 200°C and 20 kgf/cm^2 under the nitrogen gas.

Fractionation of LMSF

LMSF was fractionated by ultrafiltration membranes (UF-M) purchased from Spectrum (MWCO 100, USA) and Amicon (MWCO 500, 1000, 3000, and 10,000, USA). Molecular weight fractionated by UF-M process is not exact, generally including ± 20% errors due to an oppressive fractionation by high pressure.

Molecular weight distribution of LMSF

The molecular weight distribution of LMSF was determined by gel filtration chromatography (GFC) and MALDI-MS analysis. In the measurements of GFC, the column (2.5 × 50 cm Pyrex, Germany) was filled with Sephadex G-15 (Sigma) and swelled phosphate buffer solution. L-Glycine (MW 75), Gly-Ala (MW 146) and Gly-Tyr-Gly (MW 295) was used as the molecular weight marker kits. The absorbance at 210 nm was measured from UV detector (M720 Youngin, Korea). MALDI measurements were performed using a time-of-flight mass spectrometer system (Voyager-DE STR, Applied Biopsystems Inc., USA), operating in the positive ion linear mode. Ions formed by a pulsed UV laser beam (N_2 laser) were accelerated to 15 keV. UV laser light was focused on samples at a focal diameter of 100 – 300 μm. Lyophilized LMSF were dissolved in 0.11% dithranol (m/z 227)/tetrahydrofuran (m/z 73) solution.

Determination of the degree of inhibition (*I*) and kinetic analysis

Various concentrations of LMSF (0.8, 1.6, 2.4, and 3.2%), free amino acids, and glycine-alanine dimmer (0.2, 0.5, 0.8, and 1 M) were prepared in a phosphate buffer (pH 6.8). 1 mL of L-DOPA solution (0.6, 1.5, 1.8, 2.4, and 3 mM) was then mixed with 1.9 mL of above solutions in 5 mL vial, and followed by adding 0.1 mL of tyrosinase (0.5 mg/mL) into the mixture solution. After the solution was incubated at 25°C for 5 min, absorbance was checked at 475 nm using UV/VIS spectrophotometer (UVIKON 923, Kontron Instrument, Italy). The degree of inhibition (*I*) was expressed as percentage inhibition [$I = (1 - A_s/A_c)$ 100, where A_s and A_c were enzyme activity with and without inhibitor, respectively]. Here, ID_{50}, the concentration necessary for 50% inhibition of tyrosinase activity, was measured and kinetic properties of the inhibition by each sample were determined from Lineweaver-Burk plots of enzyme activity vs L-DOPA concentration.

Spectroscopic observation with time

Besides above samples, the solutions of reported inhibitors (0.4 and 1 mM), such as L-ascorbic acid and L-cysteine, were prepared in phosphate buffer. 0.95 mL of L-DOPA solution (1.5 mM) and 0.5 mL of each inhibitor were mixed together in UV cuvette. The reaction was initiated by the addition of 0.05 mL of tyrosinase into the mixture solution. Change in absorbance was monitored at 475 nm using UV/VIS spectrophotometer for 20 min.

Results and Discussion

Molecular weight distribution of LMSF

As a result of fractionation of LMSF using ultrafiltration membrane, it was observed that the fraction having molecular weight below 100 Da was about 40% and that between 100 and 500 Da was 50% (not shown in data), considering that the molecular weight of natural silk fibroin has been known as 300 – 500 kDa (Nahm and Shin, 1998), severe cleavage of peptide bonds occurred due to high temperature and high pressure (Kang *et al.*, 2002) and most of LMSF was remained as free amino acids or oligopeptides. It could ensure that the main species in LMSF were L-glycine, L-alanine, L-serine and

Table 1. List of amino acid and peptide species in LMSF

Compounds	Species in LMSF[1]
Free amino acids	L-glycine, L-alanine, L-serine
Dipeptides	(Gly)(Ala), (Gly)(Ser)
Tripeptides	$(Gly)^2(Ala)$, $(Gly)(Ala)^2$, (Gly)(Ala)(Ser)
Tetra and pentapeptides	$(Gly)^2(Ala)^2$, $(Gly)^3(Ala)^2$, $(Gly)^2(Ala)^3$

[1]Only main species of amino acids and peptides are listed in this Table.

Table 2. Half inhibition of tyrosinase activity (ID_{50}) values of LMSF, L-glycine, L-alanine, L-serine, and glycine-alanine peptide

Inhibitors	ID_{50}	
	Molar concentration (M)	Weight percentage concentration (%)
LMSF	-	3.4
L-glycine	0.03	0.2
L-alanine	1.24	11.0
L-serine	1.21	12.7
Gly-Ala	1.02	14.9

glycine-alanine dipeptide from the measurements of gel filtration chromatography and MALDI-MS, as listed in Table 1.

Inhibition of tyrosinase activity

For the determination of the effect of LMSF, L-glycine, L-alanine, L-serine, and glycine-alanine dipeptide on the inhibition of tyrosinase activity, ID_{50} values were compared and listed in Table 2. Here, ID_{50} means the concentration necessary for 50% inhibition of tyrosinase activity. The values were transformed into weight percentage concentration because LMSF was a complex material consisting of various free amino acids and peptides. It was found that L-glycine was the most effective compound. And LMSF also showed much higher inhibition effect compared with L-alanine, L-serine, and glycine-alanine peptide. Free amino acids and peptides inhibit the tyrosinase activity through different pathway due to different structure, composition, and molecular weight. As a result, it seams that LMSF follows all pathways of free amino acids and peptides. However, there was little difference for the inhibition effects on incubation times in all samples (data not shown), indicating no direct inhibition for tyrosinase by denaturation of the enzyme (Ben-Shalom *et al.*, 1977; Janovitz-Klapp *et al.*, 1990).

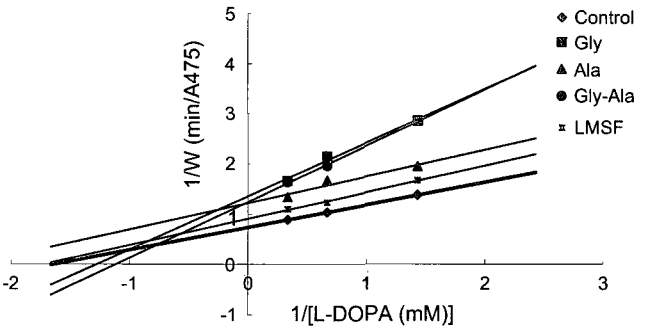

Fig. 1. Lineweaver-Burk plots for inhibition of L-glycine, L-alanine, glycine-alanine dipeptide and LMSF on mushroom tyrosinase for the catalysis of L-DOPA at 25, pH 6.8. The concentrations of L-glycine, L-alanine, glycine-alanine and LMSF are 0.5 M, 1 M, 1 M and 2%, respectively, and the concentration of enzyme is 0.5 mg/ml.

Kinetic analysis for the inhibition

Kinetic informations of tyrosinase inhibition for LMSF, L-glycine, L-alanine, L-serine and glycine-alanine peptide were obtained from Lineweaver-Burk plots (Fig. 1). The V_m values for L-glycine and glycine-alanine peptide decreased while the K_m values increased, compared with those for control, a sample without any inhibitor. However, In case of L-alanine, both V_m and K_m values increased. From these results, it can be concluded that L-glycine and glycine-alanine peptide show noncompetitive inhibitions while L-alanine follows an uncompetitive behavior (Shuler and Kargi, 1992). The kinetics of L-serine was same as that of L-alanine. On the other hand, LMSF showed mixed-type inhibition behavior and this is due to a mixture of free amino acids and peptides. Mixed-type inhibition can be observed when there are many sources for the inhibition of enzymes activity (Nerya *et al.*, 2003).

Inhibition pathway of LMSF

Free amino acids, peptides, and proteins can not only form a stable complex with metallic ions (Eichhorn, 1973; Viola *et al.*, 1979), especially Cu^{2+}, but also react with some compounds, such as quinone to have high activity (Synge, 1975).

As shown in Fig. 2, initial rate of increase of DOPAchrome decreased remarkably and the time to reach maximum absorbance increased as an increase of the concentration of L-glycine. A decrease of initial rate and an increase of the time to reach maximum absorbance can be said to mean prevention of the enzymes activity for the formation of chelates with Cu^{2+} in the enzyme. Additionally, after an arrival at maximum absorbance, the production of DOPAchrome decreased gradually, indicating the reaction of L-glycine with DOPAquinone, the precursor of

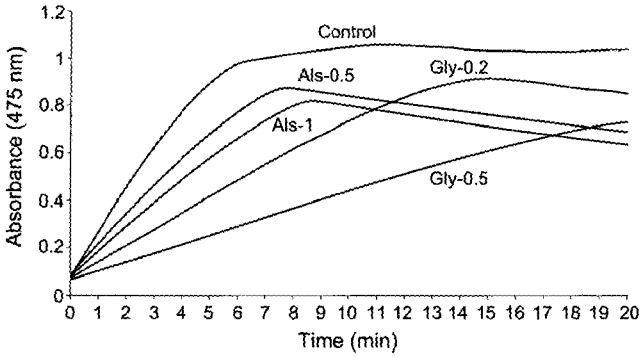

Fig. 2. Spectroscopic observation of DOPAchrome produced during the oxidation of 1.5 mM L-DOPA by mushroom tyrosinase without any inhibitor (Control) and with 0.2 M (Gly-0.2) and 0.5 M (Gly-0.5) of L-glycine and 0.5 M (Ala-0.5) and 1 M (Ala-1) of L-alanine at 475 nm.

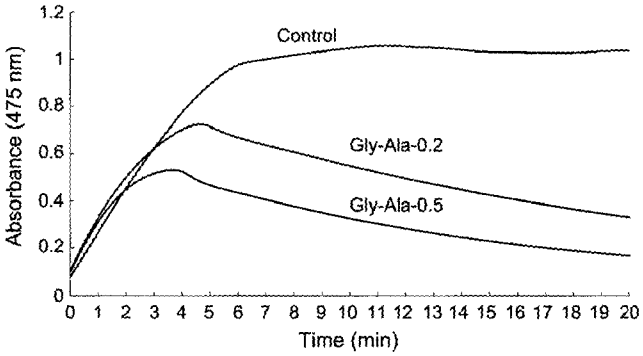

Fig. 3. Spectroscopic observation of DOPAchrome produced during the oxidation of 1.5 mM L-DOPA by mushroom tyrosinase without any inhibitor (Control) and with 0.2 M (Gly-Ala-0.2) and 0.5 M (Gly-Ala-0.5) of Gly-Ala at 475 nm.

DOPAchrome. The slope of L-alanine was somewhat similar to that of L-glycine although initial increasing rate of DOPAchrome in L-glycine was slower than that in L-alanine. It was also observed that the time to reach maximum absorbance in L-alanine was shorter than that in L-glycine and the decreasing rate of the production of DOPAchrome in L-alanine was faster than that in L-glycine. In case of L-serine, it was nearly same as Lalanine (not shown in data). These results indicate that L-glycine plays a major role in the formation of a chelate with Cu^{2+} while L-alanine and L-serine in the reaction with DOPAquinone. The reason is that the molecular weight of L-glycine is lower than that of L-alanine or L-serine, which may cause a steric hindrance. Meanwhile, although the molecular weight of L-cysteine is higher than that of L-glycine, L-alanine and L-serine, L-cysteine has a capability to form a stable complex more easily with Cu^{2+} in tyrosinase due to high affinity of thiol group with Cu^{2+} in the enzyme (Kahn, 1985).

However, when glycine-alanine dipeptide was added to the solution (Fig. 3), there was no difference of the initial increasing rate in spite of an increase of the concentration. The time to reach maximum absorbance decreased as the concentration increased and then the production of DOPAchrome after an arrival at maximum absorbance decreased sharply. These results indicate that the dipeptide of glycine-alanine could prevent the synthesis of melanin by means of not a formation of chelate with Cu^{2+} but a reaction with DOPAquinone. Fig. 4 shows the change of DOPAchrome with an addition of LMSF prepared by high-temperature and high-pressure method. Since LMSF is a mixed compound with above amino acids and peptides, it can inhibit tyrosinase activity according to the mechanisms of all components, such as L-glycine, L-alanine, L-serine, glycine-alanine dipeptide, and so on.

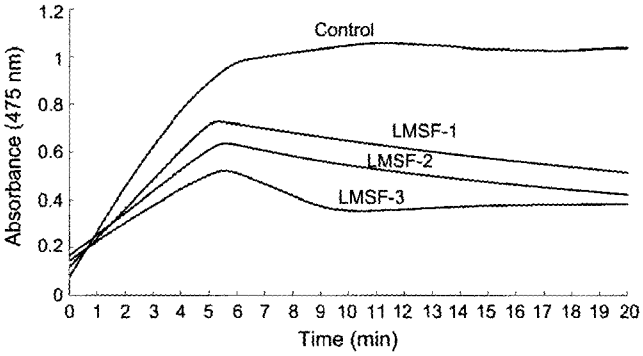

Fig. 4. Spectroscopic observation of DOPAchrome produced during the oxidation of 1.5 mM L-DOPA by mushroom tyrosinase without any inhibitor (Control) and with 1% (LMSF-1), 2% (LMSF-2) and 3% (LMSF-3) of LMSF at 475 nm. LMSF was prepared by high temperature and pressure (200, 20 kgf/cm^2) method and fractionated by ultrafiltration membrane. The molecular weight was below 100 Da.

Therefore, the initial increasing rate was slow as an increase of the concentration (a characteristic property of L-glycine or L-alanine). In addition, there was no difference of the time to reach maximum absorbance in spite of increasing concentration (the time increased in case of free amino acids while a dipeptide shortened the time). Fig. 4 also showed that the production of DOPAchrome after an arrival at maximum absorbance decreased slowly. However, lag period was not observed in any case (Fig. 2, 3 and 4), indicating that free amino acid (L-glycine and L-alanine), glycine-alanine dipeptide and LMSF could not reduce DOPAchrome to L-DOPA (Sapers and Ziolkowski, 1987; Hsu *et al.*, 1988).

Conclusively, LMSF prepared by high-temperature and high-pressure method had a specific functionality, the inhibition of tyrosinase activity. This is mainly due to the function of free amino acids and oligopeptides containing

in LMSF and the synergetic effect can be achieved by specific range of molecular weight of LMSF. Although more sophisticated fractionation of LMSF is necessary for futher studies, it may be expected that LMSF can be immediately applicable to the industry of agriculture, cosmetics and medicine fields for preventing enzymatic browning in fruits and vegetables and protecting skin from dermatological disorder, induced by tyrosinase.

References

Ben-Shalom, N., V. Kahn, E. Harel and A. M. Mayer (1977) Catechol oxidase from green olives: properties and partial purification. *Phytochemistry* **16**, 1153-1158.

Cilliers, J. J. L. and V. L. Singleton (1990) Caffeic acid autoxidation and the effect of thiol. *J. Agric. Food Chem.* **38**, 1789-1796.

Duckworth, H. W. and J. E. Coleman (1970) Physicochemical and kinetic properties of mushroom tyrosinase. *J. Biol. Chem.* **245**, 1613-1625.

Eichhorn, G. L. (1973) Inorganic chemistry. Elservier-Scientific Publishiing Co., New York.

Embs, R. J. and Na P. Markakis (1965) The mechanism of sulfite inhibition of browning caused by polyphenol oxidase. *J. Food Sci.* **30**, 753-758.

Golan-Goldhirsh, A. and J. R. Whitaker (1984) Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenyl oxidase. *J. Agric. Food Chem.* **32**, 1003-1009.

Healey, D. F. and K. G. Strothkamp (1981) Inhibition of the catecholase and cresolase activity of mushroom tyrosinase by azide. *Arch. Biochem. Biophys.* **211**, 1573-1575.

Hsu, A. F., J. J. Shieh, D. D. Bills and K. White (1988) Inhibition of mushroom polyphenoloxidase by ascorbic acid derivatives. *J. Food Sci.* **53**, 765-771.

Janovitz-Klapp, A. H., F. C. Richard, P. M. Goupy and J. J. Nicolas (1990) Inhibition studies on apple polyphenol oxidase. *J. Agric. Food Chem.* **38**, 926-931.

Jimenez, M., S. Cazarra, J. Escribano, J. Cabanes and F. Garcia-Carmona (2001) Competitive inhibition of mushroom tyrosinase by 4-substituted benzaldehydes. *J. Agric. Food Chem.* **49**, 4060-4063.

Kahn, V. (1985) Effect of proteins, protein hydrolyzates and amino acids on -dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado, and banana. *J. Food Sci.* **50**, 111-115.

Kang, G. D., K. H. Lee, B. S. Shin and J. H. Nahm (2002) Preparation and characterization of low molecular weight silk fibroin by high-temperature and high-pressure method. *J. Appl. Polym. Sci.* **85**, 2890-2895.

Maeda, K. and M. Fukuda (1996) Arbutin: mechanism of its depigmenting action in human melanocyte culture. *J. Pharmacol. Exp. Ther.* **276**, 765-769.

Mayer, A. M. and E. Harel (1979) Polyphenol oxidases in plants. *Phytochemistry* **18**, 193-215.

Nahm, J. H. and B. S. Shin (1998): . The structure and physicochemical properies of silk; in *Silk science.* Sunwoo, J. H. (ed.), pp. 84-99, SNU Press.

Nerya, O., J. Vaya, R. Musa, S. Izrael, R. Ben-Arie and S. Tamir (2003) Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. *J. Agric. Food Chem.* **51**, 1201-1207.

Richard-Forget, F. C., P. M. Goupy and J. J. Nicolas (1992) Cysteine as a inhibitor of enzymatic browning. 2. kinetic studies. *J. Agric. Food Chem.* **40**, 2108-2113.

Robert, C., C. Rouch and F. Cadet (1997) Inhibition of palmito (*Acanthophoenix rubra*) polyphenol oxidase by carboxylic acids. *Food Chem.* **59**, 355-360.

Sapers, G. M. and M. A. Ziolkowski (1987) Comparison of erythobic acid and ascorbic acids as inhibitors of enzymatic browning in apple. *J. Food Sci.* **52**, 1732-1733.

Sayavedra-Soto, L. A. and M. W. Montgomery (1986) Inhibition of polyphenoloxidase by sulfite. *J. Food Sci.* **51**, 1531-1536.

Shuler, M. L. and F. Kargi (1992) Enzymes; in *Bioprocess engineering basic concepts.* F. Kargi (ed.), pp. 58-102, Prentice Hall PTR, New Gersey.

Sugimoto, K., T. Nishimura, K. Nomura, K. Sugimoto and T. Kuriki (2003) Syntheses of arbutin--glycosides and a comparison of their inhibitory effects with those of -arbutin and arbutin on human tyrosinase. *Chem. Pharm. Bull.* **51**, 798-801.

Synge, R. L. M (1975) Interaction of polyphenols with proteins in plants and plant products. *Plant Foods Hum. Nutr.* **24**, 337-340.

Taylor, S. L. and R. K. Bush (1986) Sulfites as food ingredients. *Food Technol.* **40**, 47-52.

Viola, R. E., C. R. Hartzell and J. J. Villafranca (1979) Copper(II) complexes of carnosine, glycylglycine, and glycylglycine-imidazole mixtures. *J. Inorg. Biochem.* **10**, 293-307.