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Antioxidant activity of silkworm powder treated with protease

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Abstract

The antioxidant activity of silkworm powder treated by proteolytic enzyme was investigated. Total protein content of silkworm power was assayed using BCA, Bradford assays and SDS-polyacrylamide gel electrophoresis (PAGE) with alkaline protease treatment conditions including temperature and pH. The optimum condition of alkaline protease treatment for silkworm powder was found to be 60°C and pH 7. The alkaline protease treatment resulted in increased contents of free amino acids, total polyphenol and total flavonoid compared to control group. The silkworm hydrolysates showed excellent antioxidant activities in various *in vitro* models such as 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2 –azino-bis(3-ethylbenzthiazoline-6)-sulfonic acid (ABTS) radical scavenging activity. These results provide useful information for using silkworm powder as an ingredient in functional foods and for exploiting alkaline protease treatment to improve the extractability and bioactivity of a raw material.

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Introduction

The silkworm, *Bombyx mori*, is a monophagous phytophagous lepidopteran species, feeding only on mulberry leaves. There was used the cocoon production and medicine for a long history in Korea, Japan and China. There are many varieties of *Bombyx mori*, with more than 300 *B. mori* varieties in South Korea. Recently, the dress-centered textile business was moved into the advanced bio-industry emphasizing a useful composition of silkworm products. One of such a new application of silkworm products is utilization of such resources itself from the production to health functional food, medical source, biotech application and so on (Lee and Kim, 2000; Jo *et al*., 2012; Foss *et al*., 2013; Kweon *et al*., 2014; Ju *et al*., 2014).

There are five instars during the silkworm larval phase. After freezing desiccation, and porphyrization, silkworm larvae on the third day of the fifth instar can be processed to be silkworm larvae powder. It can be easily digested and absorbed by human bodies. The general composition of silkworm larvae contains 10.6% water, 54.8% crude protein, 9.4% crude fat and 7.2% crude fiber (Kim *et al*., 2008). The silkworm larvae and silkworm products are also rich in useful components (Ryu *et al*., 2012; Chon *et al*., 2013; Ju *et al*., 2014; 2015). These abundant nutrient elements can promote the physiological functions in human and animal health. Many researchers reported that the silkworm larvae powder have a sub-medicine to blood LDL-cholesterol lowering

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effects (Kim, 2008), antioxidant effects (Choi *et al*., 2000), and antihepatotoxic effects (Kim *et al*., 2008). Negative effects on human health have not been reported yet.

In general, the physical, chemical and enzymatic methods used to improve the low functionality of the protein. Among them, protein hydrolysis by enzymatic method is considered as a powerful method to wide new functional and physiological activities of protein. Alkaline proteases are most widely used in industries. Other than traditional uses in a variety of industries, meat tenderization, cheese-making, dehairing, baking, brewery, the production of digestive aids (Singhal *et al*., 2012). It was produced by a wide range of organisms, including bacteria, fungi, yeasts, and mammalian tissues. Recently, a large amount of commercial alkaline proteases are derived from strains of *Bacillus* (Mabrouk *et al*., 1999; Mehrotra *et al*., 1999). In recent years, interest in health functional food of silkworm powder is increasing in Korea. The aim of the present study was to investigate the biological activity and physiochemical properties effects of silkworm powder by treatment with alkaline protease.

Materials and Methods

Preparation of silkworm powder extract

Silkworm larvae (*Bombyx mori*) were reared by feeding mulberry leaves at the National Institute of Agricultural Science. The silkworm varieties used for the experiment was Kumokjam. The larvae of 3rd of the 5th instar were quickly frozen with in liquid nitrogen and lyophilized. FoodPro Alkaline Protease was obtained from Bision Corporation (SeongNam, Korea) and stored at 4 ºC until use. For the enzymatic treatment, 1 g of silkworm powder was used as a substrate: 10 mL of distilled water and 10 uL of alkaline protease were added to the conical tube containing the powder.

The effect of temperature and pH on the protease activity was calculated at various temperatures (30, 40, 50, 60, and 70 ºC at pH 7) and pH (3, 5, 7, 9, and 11 at 60 ºC) with either 1 M HCl or 1 M NaOH. The reaction mixture was conducted on a shaking incubator (150 rpm) for 24 h. After this period, the reaction mixture was incubated 90 ºC for 15 min to inactivate the alkaline protease, and the mixture was filtered through filter paper. The filtered sample was centrifuged at 13,000 rpm for 10 min and the supernatant was used for experimentation.

Measurement of protein concentration

The silkworm powder extracts have been determined by two colorimetric methods, namely the Bradford and bicinchoninic acid (BCA) assays. Bradford assay measures proteins by their binding capacities to Coomassie Brilliant Blue (CBB) to form a protein-dye complex. This interaction shifts the peak absorbance of the dye from 465 nm to 595 nm, resulting to change of the solution color from red-brown to blue (Bradford, 1976). All measurement samples were diluted 100-fold. The procedure was performed as described previously (Bradford, 1976) using Bradford reagent (Sigma-Aldrich, USA). BCA assay combines the reduction of Cu^{2+} to Cu^{1+} by peptide bonds of the protein in an alkaline solution with the selective colorimetric reaction of BCA-Cu^{1+} to form purple complex, which is strongly absorbed at 562 nm (Smith *et al*., 1985). All measurement samples were diluted 1000-fold, and used a BCA protein assay kit (GenDEPOT, USA). BCA assay was performed according to the manufacturer's protocol. All measurements were conducted in triplicate.

SDS-PAGE analysis

SDS-PAGE of the alkaline protease treated silkworm powder was performed according to the method of Laemmli (Laemmli., 1970) under denaturing conditions on a 4-12% gradient gel. Aliquots of extract was separately dissolved in NuPAGE LDS 4x Sample Buffer (Invitrogen, USA) and boiled for 10 min. The sample solutions were centrifuged at 13,000 rpm for 10 min and the supernatants were loaded into the parallel gel wells. After carrying out the electrophoresis, the separated proteins were visualized by Coomassie brilliant blue G-250 staining. A prestained protein ladder (Bio-Rad, USA) was used as standard molecular mass markers.

Total polyphenol content

The total phenolic content was estimated by Folin Ciocalteu method as described by Singleton and Rossi (1965) with slight modifications. The extract was mixed with 8 mL of distilled water, 1 mL of sodium carbonate (15%) and 1 mL of Folin & Ciocalteu phenol reagent (1N). The mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer. The reference compound was used catechin.

Total flavonoid content

The flavonoids content was determined by aluminium trichloride method using quercetin as reference compound (Zhishen *et al*., 1999). A volume of 125 μL of extract is added to 75 μL of a 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min, and then 150 μL of aluminium trichloride (10%) was added and incubated for 5 min, followed by the addition of 1000 μL of NaOH (1M). The final volume of the solution was adjusted to 2000 μL with distilled water. After 15 min of incubation the mixture turned to pink and the absorbance was measured at 510 nm.

Determined of free amino acids concentration

Amino acid content in the freeze-dried extract samples was determined based on previously reported methods (Li *et al*., 2011). The amino acids were determined in triplicate using the Amino Acid Analyzer (L-8900, HITACHI, Japan), and results were presented milligram of amino acids per 1 milligram of freeze-dried extract samples (mg/mg).

DPPH radical reduction assay

The assay was carried out as previously described (Hatano *et al*., 1988). Each 100-fold diluted sample (100 uL) was added to 100 uL of 0.4 mM 2,2 dipheny l-1-picrylhydrazyl (DPPH) in methanol. The mixture was shaken vigorously and allowed to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. All measurements were conducted in triplicate. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

DPPH radical scavenging activity (%) = (Abs control −Abs sample) / Abs control

ABTS scavenging activity assay

Total antioxidant status of the protease treated silkworm powder was measured using 2,2′-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) assay (Re *et al*., 1999). ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12~16 h) in the dark before use. The resultant intensely-coloured $ABTS^{+}$ radical cation was diluted with 0.01 M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. 10 μL of 100-fold diluted samples were added to 190 μL of ABTS solution in a well of 96-well plate, and the absorbance were recorded at 734 nm after 10 min incubation at room temperature. All measurements were conducted in triplicate. The percentage of the ABTS radical scavenging is calculated using the equation as given below:

ABTS radical scavenging activity (%) = (Abs control −Abs sample) / Abs control

Results and Discussion

Determination of hydrolysis optimum condition

The effect of temperature and pH on silkworm powder by alkaline protease treatment was assayed for their total protein content using both BCA and Bradford assays calibrated against bovine serum albumin (BSA). Results obtained on both as non-treated and treated alkaline protease are summarized in Fig. 1. The BCA assay, the amount of protein was increased after treatment of alkaline protease. Otherwise, the silkworm powder protein was significantly decreased using Bradford assay. The optimum temperature and pH of alkaline protease for hydrolysis of silkworm powder protein was found to be 60 ºC and pH 7 using BCA assay (Fig. 1A, 1B). In addition, silkworm powder protein was degraded most by both 60 ºC and pH 7, 9 using Bradford assay (Fig. 1C, 1D). Bradford assay measures proteins by their binding capacities to Coomassie Brilliant Blue (CBB) to form a protein-dye complex. This interaction shifts the peak absorbance of the dye from 465 nm to 595 nm, resulting to change of the solution color from red-brown to blue. The previous reported indicating that Bradford assay was very sensitive and suitable for the sample with low protein concentrations such as the urine (McElderry *et al*., 1982). However, it was not applicable for peptide and amino acid measurement (Chutipongtanate *et al*., 2012). Principle of CBB stained SDS-PAGE is similar to protein quantitative analysis by Bradford assay. Our result, also, showed that SDS-PAGE analysis was similar to those obtained by Bradford assay (Fig. 2). Generally, basic subunits were less easily digested than acidic subunits. We considered that the silkworm powder was degraded into peptide and free amino acids by alkaline protease. Collectively, our analyses suggest that the optimum temperature

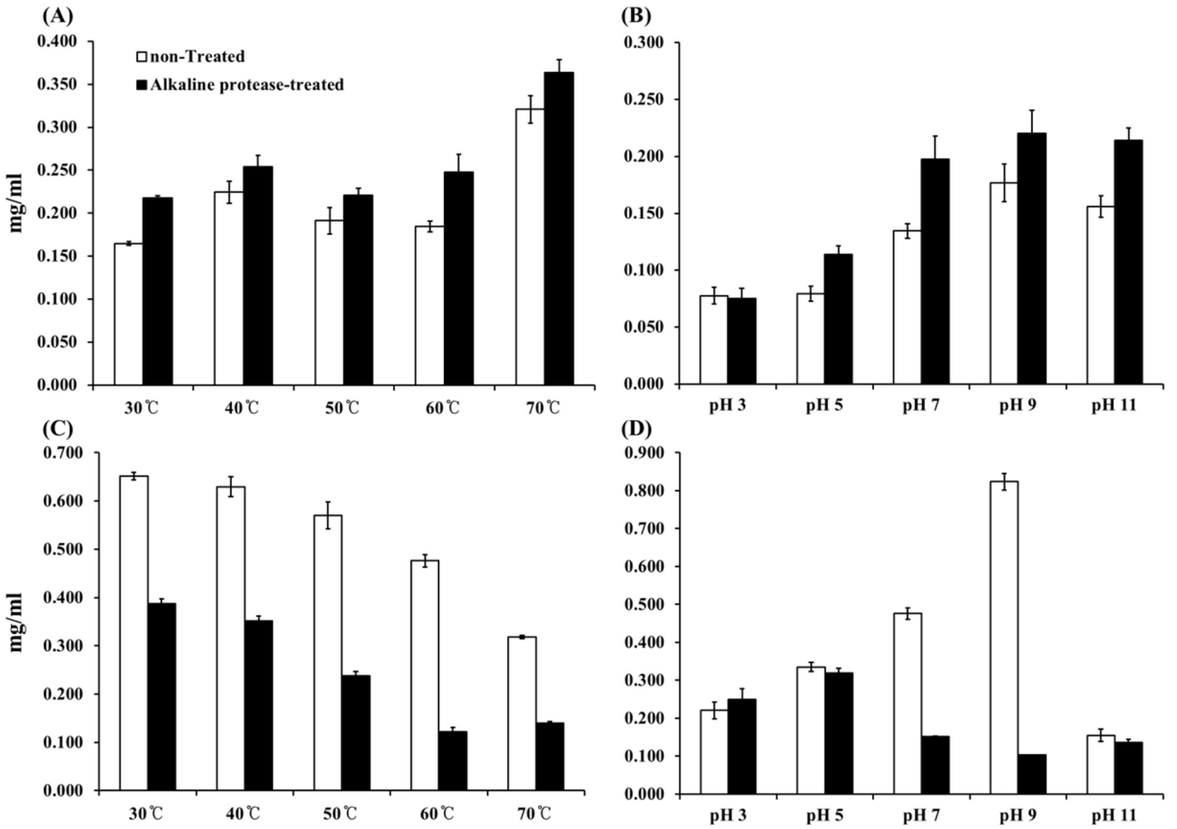

Fig. 1. Quantitative analysis on the silkworm powder extracts by the alkaline protease treatment. Total protein of the silkworm powder extracts was measured using two colorimetric methods, namely the BCA (A, B) and bradford (C, D) assays. The white boxes were silkworm powder of treatment without alkaline protease, and the black boxes were silkworm powder of treatment with alkaline protease. The bars indicate the mean ± SE. All measurements are the mean of three experimental data.

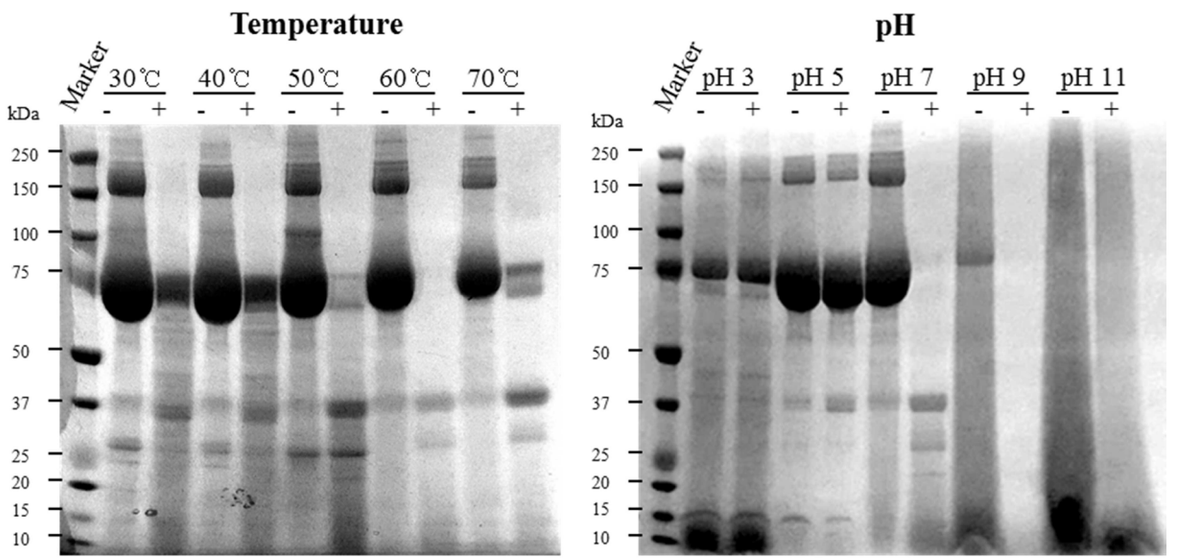

Fig. 2. SDS-PAGE analysis of silkworm powder after proteolytic digestion. SDS-PAGE was performed for silkworm powder after treatment without (-) or with (+) alkaline protease for 24 h at various temperature and pH. Reaction products and controls stained by coomassie brilliant blue.

and pH of treatment alkaline protease for silkworm powder was found to be 60 ℃ and pH 7.

Free amino acids and total antioxidant compounds contents

The amino acids represent our largest group of mutually analogous nutrients. Correspondingly wide is their representation among metabolites and body components. We demonstrated that the changes in extractability of the silkworm powder with or without protease treatment measured by analyzing the amounts of free amino acid after treatment (Fig. 3). The protease treated silkworm powder had increased all kinds of free amino acid composition amount. Especially, the compositions of glutamic acid, aspartic acid were higher in the non-treated silkworm powder than those in the protease treated silkworm powder. Free amino acid composition is highly correlated with protein denaturation, which involves a change in protein structure. Amino acids are joined together by peptide bonds, and additional bonds are contributed to form the protein into convoluted shapes between amino acids. Antioxidant activities of free amino acids have been evaluated (Hernandez-Ledesma *et al*., 2005) and a number of amino acids have been proposed to contribute positively to the antioxidant activity of purified food-derived and synthetic natural peptides. However, the chemistry and mechanisms of action have not been studied in detail. Previously report showed that the glutamic acid and aspartic acid can influence their strong contributions to the antioxidant activities (Udenigwe and Aluko, 2011). We suggested that the alkaline protease might impact the additional bonds between each amino acid resulting in changes in the amount of free amino acid in silkworm powder.

We also investigated changes in the total polyphenol and flavonoid contents of silkworm powder following protease treatment (Table 1). The protease treated silkworm powder extract significantly increased polyphenol and flavonoid contents. Many reports have described that the physiological function of natural foods can be attributed to the antioxidative capacity of their antioxidant components (Ness and Powles, 1997; Halliwell, 1999). We considered that protease treatment was increased polyphenol and flavonoid release from the silkworm powder. Enzymatic hydrolysis is shown to influence the emulsifying and foaming properties of proteins, thus affecting their utilization as food ingredients (Adler-Nissen, 1983). The protease may have acted by catalyzing the hydrolytic degradation of intracellular organelles and cell membranes of protein nature, such as e.g. the tonoplast surrounding the cell vacuoles or the extensions forming the cell wall.

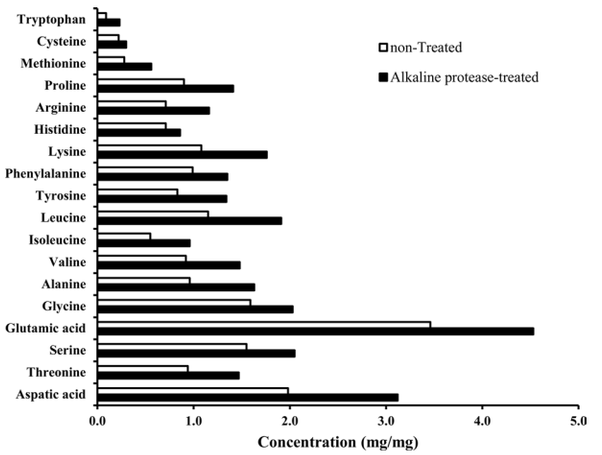

Fig. 3. Measurement of free amino acid in silkworm powder hydrolysate. Individual free amino acid composition analysis. Individual free amino acid composition analysis also showed difference between the non-treated (open columns) and alkaline protease-treated (filled columns) silkworm powder.

Table 1. Total phenolics and flavonoids content of alkaline protease treated silkworm powder

	Total Polyphenol	Total Flavonoid
non-Treated	150.16	22.09
Alkaline protease-treated	202.59	36.09

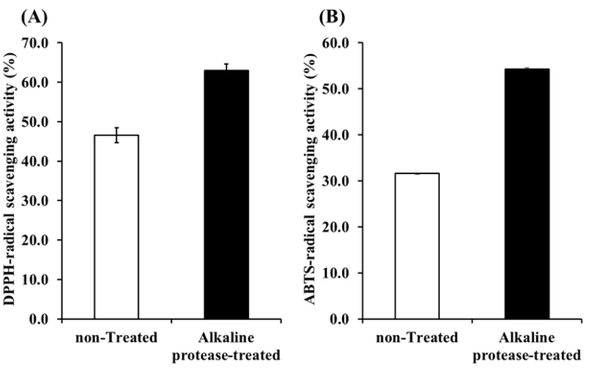

Fig. 4. Scavenging effect of silkworm powder hydrolysates on DPPH (A) and $ABTS^{+}$ radicals (B). The white boxes were silkworm powder of treatment without alkaline protease, and the black boxes were silkworm powder of treatment with alkaline protease. (n=3, mean ± SE). Error bars represent standard error from triplicate determinations.

Antioxidant activity of silkworm powder hydrolysate

Measurement of antioxidant capacity using single method cannot provide the compound's ability to act as antioxidant. Hence, this study involved two in vitro methods such as DPPH radical scavenging activity, $ABTS^+$ radical scavenging activity. When DPPH and $ABTS^+$ radicals encounter a proton-donating substance such as an antioxidant, the radical is scavenged, and the absorbance is reduced by changing the color (Liu *et al*., 2010). The universally used parameters of free radicals' scavenging ability as well as the evaluation of antioxidant potency are reactions with the radical cation $ABTS^+$ and stable radical DPPH (Miller and Rice-evans, 1996; Sanchez-moreno *et al*., 1998). The $ABTS^+$ method is a useful tool in determining the antioxidant activity of both lipophilic (e.g. alpha-tocopherol or beta-carotene) and hydrophilic antioxidants in various matrices (body fluids, food extracts, etc.) (Cano *et al*., 2000). As described in Fig. 4, silkworm powder by protease treatment showed excellent DPPH and $ABTS^+$ radical-scavenging activity. The results agree with previous reports that indicate hydrophobic amino acids and antioxidant composition are able to interact better that contain these free radicals (Udenigwe and Aluko, 2011; Sharma *et al*., 2015). Wang *et al*. (2010) suggested that enzymatic hydrolysis products with optimal enzymes and high protease and peptidase activities showed higher antioxidant activity compared to those of a non-treated control due to their ability to improve extractability from the phenolic-protein complex. Additionally, mulberry leaves have been used to feed Silkworms. Previously reported that the mulberry leaves have numerous bioactive components including 25 kinds of amino acids and functional antioxidant composition (Shin, 1998; Kodama *et al*., 1990; Chae *et al*., 2003). As a result, it increased the antioxidant activity of the silkworm powder by protease treatment. Application of proteolytic enzymes is often an attractive means for improving functional properties of food proteins, without losing their nutritional and biological value.

In conclusion, these findings demonstrate that usefulness of silkworm powder by protease treatment containing functional composition results in enhancement of antioxidant activity. The results also suggested the silkworm powder maybe have potential application in food engineering.

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