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Antioxidant activity of chemically and enzymatically modified sericin extracted from cocoons of *Bombyx mori*

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ABSTRACT

Thai silk cocoons from polyvoltine silkworm strain, Nangnoi, contain several antioxidant factors, pigments and protein. The present study investigated relationship between sericin protein treated with DTT, β-mercaptoethanol, UV light and protease enzyme and antioxidant activity of resultant sericin protein. The antioxidant activity of crude sericin extract (CSE) treated with protease enzyme measured by ABTS and DPPH assays showed 5.0-fold and 3.0-fold higher activity than that of untreated CSE, while DTT, β-mercaptoethanol and UV light treatments were found to significantly decrease antioxidant activity of CSE. Then, CSE was fractioned by salting out. The colorless supernatant (SNT) fraction showed the highest antioxidant activity. In addition, the antioxidant activity of fractionated sericin protein treated with various treatments showed similar results to those of similarly treated CSE. The results obtained in this study suggest that sericin protein modified using protease enzyme could be adopted as ingredients in food and cosmetic applications.

1. Introduction

There are a large number of polyvoltine silkworm strains which produce Thai silkworm cocoons. The individual strain exhibits unique properties such as cocoon shape, color, total cocoon weight, and antioxidant activity (Chlapanidas et al., 2013). For Thai polyvoltine silkworm cocoons, they are composed of proteins and pigments (carotenoids and flavonoids) (Tabunoki et al., 2004; Tamura et al., 2002). Pigments are commonly known for their biological properties, including antioxidant activity (Heim et al., 2002; Khanam et al., 2012; Wu et al., 2015). Silk protein is composed of two protein components, fibroin and sericin. Sericin is a family of cocoon proteins specifically synthesized in the middle silk gland of the silkworm *Bombyx mori*, constitutes about 20–30% of total cocoon weight (Zhang, 2002). Sericin has been known to possess biological functions, such as antioxidant and anti-tyrosinase activities (Aramwit et al., 2010; Kato et al., 1998).

Recently, use of natural protein extract or purified proteins as antioxidant has attracted particular interest. Many food proteins from milk, soy bean, fish and corn were reported to have antioxidant activity (Chalamaiah et al., 2012; Pihlanto, 2006; Ranamukhaarachchi et al., 2013; Wang et al., 2014). Several studies have shown that protein hydrolysate and peptides could be used as alternative antioxidants (Guo et al., 2009; Zhang et al., 2009; Sarmadi and Ismail, 2010; Ghribi et al., 2015). Moreover, there are reports that chemical and physical treatments (dithiothreitol (DTT), UV light) increased antioxidant activity of albumin, whey protein and chickpea protein (Li et al., 2008; Medina-Navarro et al., 2010; Chen et al., 2012). DTT, UV light and protease enzyme could be induced conformational and functional changes of proteins, including antioxidant activity. The antioxidant activity of proteins mostly depends on their structure and amino acid composition. However, no studies are present on effect of some chemical and physical treatments on the structure of sericin protein and on antioxidant activity, particularly from *B. mori* silkworm strain, Nangnoi.

In this study, sericin from cocoons of polyvoltine silkworm strain, Nangnoi, was extracted and fractionated by salting out, and modified with DTT, β-ME, UV light exposure and hydrolysis by protease enzyme to investigate the relationship between sericin protein and its antioxidant activity.

2. Materials and methods

2.1. Materials and chemicals

The yellow cocoons of the silkworm, *B. mori*, strain, Nangnoi,

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were obtained from Silk Innovation Center, Mahasarakham University, Thailand. The chemicals 2,2-diphenyl-1 picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulfonic acid) (ABTS), dithiothreitol (DTT), β-mercaptoethanol (β-ME) and protease from *Streptomyces griseus* (Type XIV, $\geq$3.5 units/mg solid, E. C. 232-90-5) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Standard protein molecular weight marker was purchased from GeneDirex Inc., Taiwan. Ammonium sulfate [$(NH_4)_2SO_4$] was purchased from Carlo Erba, Italy. All other chemicals and reagents used in the study were of analytical grade.

2.2. Sericin extraction

Silk cocoons (2 g) were degummed in 200 ml of distilled water with different extraction time for 15, 30, 60, 90 and 120 min at 98 °C to obtain crude sericin extracts (CSE). Protein concentration and antioxidant activity of CSE were determined by Bradford assay and ABTS and DPPH assays, respectively.

2.3. Protein determination by Bradford assay

Sericin extract was diluted with distilled water. Sample (0.5 ml) was added to 1.0 ml of Bradford solution and incubated at room temperature for 5 min. Bovine Serum Albumin (BSA) was used as a standard reference protein. The absorbance of samples was measured at 595 nm with visible spectrophotometer (Thermo Spectronic, U.S.A.).

2.4. Protein fractionation by salting-out with saturated $(NH_4)_2SO_4$

Saturated $(NH_4)_2SO_4$ was added to CSE (10.0 ml) until whole yellow colored-protein precipitated. Then, it was centrifuged at 8000 g for 5 min to separate yellow-precipitate (Yellow-PT) and supernatant (SNT). The Yellow-PT was re-dissolved in distilled water and both samples were then dialyzed against distilled water for 5 h. Protein concentration was measured by Bradford assay and antioxidant activity was measured by ABTS and DPPH assays.

2.5. Treatment with dithiothreitol (DTT) and β-mercaptoethanol (β-ME)

CSE (50.0 ml) and SNT were treated with DTT. Samples containing 1200 μg/ml of protein were incubated with 0.5, 1, 5, 10 and 20 mM of DTT for 10 min at room temperature and then dialyzed against distilled water for 24 h to obtain the CSE and SNT treated with DTT. Protein concentration of the samples was determined by Bradford assay and antioxidant activity of the samples was determined by ABTS and DPPH assays. Treatment with β-ME was performed similarly to treatment with DTT except concentration of β-ME at 1, 5, 10, 20 and 30 mM to obtain the CSE and SNT treated with β-ME.

2.6. Treatment with UV light

CSE, Yellow-PT and SNT were exposed to UV light (254 nm) at a distance of 15 cm from a source lamp for 35 Section, 3, 5 and 10 min at room temperature to obtain the CSE, Yellow-PT and SNT after to UV light. Antioxidant activity of the samples was determined by ABTS and DPPH assays.

2.7. Treatment with protease

CSE, Yellow-PT and SNT were treated with protease enzyme at 37 °C pH 7.5 for 3 h. The enzyme-to-substrate protein ratio was 4:100 (w/w) (Wu et al., 2008). The pH of the sample solutions was adjusted for enzymatic hydrolysis with 0.1 mM NaOH. The enzymatic reaction was stopped by heating at 90 °C for 20 min to obtain the CSE, Yellow-PT and SNT treated with protease. Protein concentration of the samples was determined by Bradford assay and antioxidant activity of the samples was assayed by ABTS and DPPH assay. Molecular mass of the samples was estimated by SDS-PAGE.

2.8. Antioxidant activity by ABTS assay

Experiments were performed according to Re et al. (1999) with slight modifications. ABTS and potassium persulfate were dissolved in distilled water to a final concentration of 7 mM and 2.45 mM, respectively. These two solutions were mixed and the mixture was allowed to stand in the dark at room temperature for 16 h before using in order to produce ABTS radical cation ($ABTS^{\bullet+}$). For the study of antioxidant activity, $ABTS^{\bullet+}$ solution was diluted with distilled water before using. Samples were diluted in distilled water and added into 1 ml of ABTS solution. The sample was mixed and incubated in the dark at room temperature for 6 min. Then absorbance was measured at 734 nm. The total antioxidant capacity was expressed as percent inhibition, according to the equation: Percent inhibition = [1 − (Abs. sample/Abs. control)] × 100. The values were expressed as an IC_{50} value, which is the concentration of the extract that scavenges 50% of the $ABTS^{\bullet+}$

2.9. Antioxidant activity by DPPH assay

The DPPH radical scavenging activity of samples was measured according to a slightly modified method (Yamaguchi et al., 1998). Samples were subjected to determine the least concentration of radical scavenging. Each sample solution (0.5 ml) was added to 1 ml of a freshly prepared 0.1 mM DPPH solution dissolved in methanol. The sample was mixed and incubated in the dark at room temperature for 30 min. Absorbance of the sample was measured at 517 nm using a spectrophotometer against a control. The radical scavenging of DPPH was calculated by using the formula below: Radical scavenging activity (%) = [1 − (Abs. sample/Abs. control)] × 100. Then results were expressed as an IC_{50} value, which is the concentration of the extract that scavenges 50% of the DPPH radical scavenging activity.

2.10. Estimation of molecular mass of sericin proteins by SDS-PAGE

The molecular mass of sericin proteins was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separating gel and staking gel were 12.5% and 5%, respectively. The sample solution was mixed with 2x buffer and heated in hot water, then sample was loaded into the well. The electric current of 150 V, 50 mA was applied to the gel. At the end of electrophoresis, the gel was stained with silver staining technique. Standard molecular mass marker was applied for estimating the molecular mass.

2.11. Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). One-way ANOVA using SPSS software was used to compare the mean values of each treatment. Significant differences ($p < 0.05$) among the means were determined by using Duncan's multiple range Test.