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Original Research Article

Development of drop test immunoassay for melatonin detection

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ABSTRACT

Melatonin (N-acetyl-5methoxytryptamine) plays an important role in the regulation of sleep cycles, immune functions and antioxidant capabilities. Various methods are used to determine melatonin concentration such as high-performance liquid chromatography (HPLC). However, this techniques is high-cost and not suitable for analyzing colloid samples such as milk. Enzyme-linked immunosorbent assay (ELISA) can be used for colloid samples but this is also expensive and time consuming. Thus, a fast and reliable method for melatonin detection was developed. Immunogenic melatonin was prepared by coupling melatonin with carrier protein as bovine serum albumin (BSA) using the Mannich reaction. After covalently bound with BSA, the melatonin-BSA coupling (Mel-BSA) was then used for rabbit immunization. The purified antibody was selected to construct a drop test immunoassay for melatonin detection. Under optimized condition, the limit of detection was shown to be 0.15 ng/mL with a turnaround time of about 20 minutes. This drop test immunoassay is a promising approach for development of a more rapid, robust and cost-effective tool for melatonin detection.

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INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous hormone secreted by the pineal gland in the center of the brain. In mammals, this hormone plays an important role in physiological functions such as the regulation of dark-light signal transduction, immune function, and blood pressure (Pévet, 2002). Melatonin levels in serum are age-dependent whereby younger serum has a higher level. Melatonin is used to treat older adults with sleeping disorders together with other pharmaceutical applications including treatments for insomnia, heart disease, menopause, anti-aging, and cancer (Malhotra et al., 2004).

High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) are both used to determine melatonin levels (Lin et al., 2012). However, these methods are expensive and not suitable for analyzing colloid samples such as milk. Enzyme-linked immunosorbent assay (ELISA) is a highly specific and sensitive technique which can be used to detect melatonin in colloid samples, but it is time consuming and costly (Li and Cassone, 2015). Thus, here, a fast and reliable method to detect melatonin in all types of food samples was developed, with a turnaround time of no more than 20 minutes.

MATERIALS AND METHODS

Preparation of melatonin conjugated BSA (Mel-BSA)

Solution A was prepared by dissolving 150 mg of BSA in 5 mL of distilled water. Solution B was prepared by dissolving 50 mg of melatonin in 5 mL of distilled water and ethanol in the ratio 2:1 (v/v). Both solutions were mixed gently and then 3 mL of 3 M sodium acetate and 5 mL of 7.5% formaldehyde were added. The mixture was incubated and stirred for 24 hours in the dark. Mel-BSA was dialyzed with 0.05 M phosphate buffer, pH 7.4 overnight and then filtered through Whatman No. 1 filter paper (Soukhtanloo et al., 2008; Yang et al., 2006). The Mel-BSA was determined using Fourier-transform infrared spectroscopy (FTIR), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, scanning electron microscope (SEM) and HPLC.

Fourier-transform infrared spectroscopy

Samples of BSA, melatonin, and Mel-BSA were analyzed by an HTS-XT Tensor 27 Spectrophotometer (Bruker, Marne-la-Vallée, France). Each analysis was replicated 3 times and the average spectrum was used to interpret the functional groups using http://www.science-and-fun.de/tools/ (Swann and Patwardhan, 2011).

Scanning electron microscopy

Samples of BSA, melatonin, and Mel-BSA were dried in a centrifugal concentrator (Tomy, Japan). Following drying, the samples were coated with gold using Balzers SCD-040 and examined using a Scanning Electron Microscope JSM-6420.

HPLC analysis

Melatonin and Mel-BSA were analyzed using the HPLC technique of Lin et al. (2012) with slight modifications. The mobile phase was methanol: acetonitrile: 0.5% acetic acid solution (4:1:5, v/v/v) at a flow rate of 0.5 mL/min, measured at a wavelength of 250 nm. Injection volume was 10 µL. Melatonin dissolved in methanol at concentrations ranging from 0.2-0.6 mg/mL was used as reference. All samples were dissolved in methanol and filtered by 0.45 µm and 0.20 µm organic filter membranes before assay.

Mel-BSA antibody production

Polyclonal antibody against Mel-BSA generated here was raised in rabbits. Two rabbits (New Zealand White, female) were used to ensure that at least one seroconverted. A day prior to immunization (day 0), a pre-bleed was collected as a control. The first injection was prepared as a mixture of protein antigen and complete Freund's adjuvant (Sigma, USA) at 1:1 ratio, vortexed until it emulsified, and then intramuscularly and subcutaneously injected. The second, third, and fourth injections were carried out subsequently every 2 weeks but with incomplete Freund's adjuvant (Sigma, USA). Approximately 100 µg of protein antigen in 0.5-1.0 mL was injected per rabbit. Blood samples were collected on days 35 and 49 after the first injection to observe specificity and titer of polyvalent anti-Mel-BSA. Immunization was carried out by Genscript, USA (a qualified home office license holder).

Drop test immunoassay

Around 5 µg of Mel-BSA antibody and anti-Rabbit Ab were dropped on a nitrocellulose membrane and then dried in a desiccator at room temperature for 30 minutes. The membrane was soaked in blocking buffer (PBST pH 7.4, 5% (w/v) Casein). After drying, all samples were added. The assay was read by the color of colloidal gold on the membrane using ImageJ software (National Institutes of Health, USA).

RESULTS AND DISCUSSION

Fourier-transform infrared spectroscopy (FTIR)

The FTIR results (Figures 1-3) showed that the structure of BSA consisted of N-H stretching, C=O stretching and N-H bending at wavenumbers 3600-3000, 1690-1650 and 1550-1510 cm-1, respectively. The melatonin structure consisted of N-H stretching and C-H bending (-CH3) at wavenumbers 3600-3000 and 1450-1375 cm-1, respectively. The FTIR spectra of Melatonin was similar to what has been described by Bongiorno et al. (2004). The structure of Mel-BSA consisted of N-H stretching, C-H bending (-CH2) and C-H bending (-CH3). These results indicated that melatonin was covalently bound with BSA by Mannich reaction. Therefore, this Mel-BSA can be used for rabbit immunization to produce polyclonal antibodies against melatonin.

Scanning electron microscopy (SEM)

SEM was carried out for surface assessment (Fischer *et al*., 2012). The 3D structure of BSA was determined at 100x magnification, while melatonin and Mel-BSA were assessed at 750x magnification. Results suggested that the surface of Mel-BSA had combined characteristics of both melatonin and BSA (Figure 2).

HPLC analysis

The HPLC chromatogram (Figure 3) shows retention times of standard melatonin and Mel-BSA at 11.165 minutes and 11.193 minutes, respectively. Concentration of melatonin in Mel-BSA was estimated to be 0.01 mg/mL.

Anti Mel-BSA sera

Since the molecular weight of melatonin is very small (~232 g/mol), the size of Mel-BSA was similar to BSA (Soukhtanloo *et al*., 2008; Pubchem, 2018) when assessed with SDS-PAGE as shown in Figure 4A. Pre-absorption purified Mel-BSA antisera with 5% BSA were shown as an efficient way to resolve cross-reactivity of the antisera to BSA (Figure 4B).

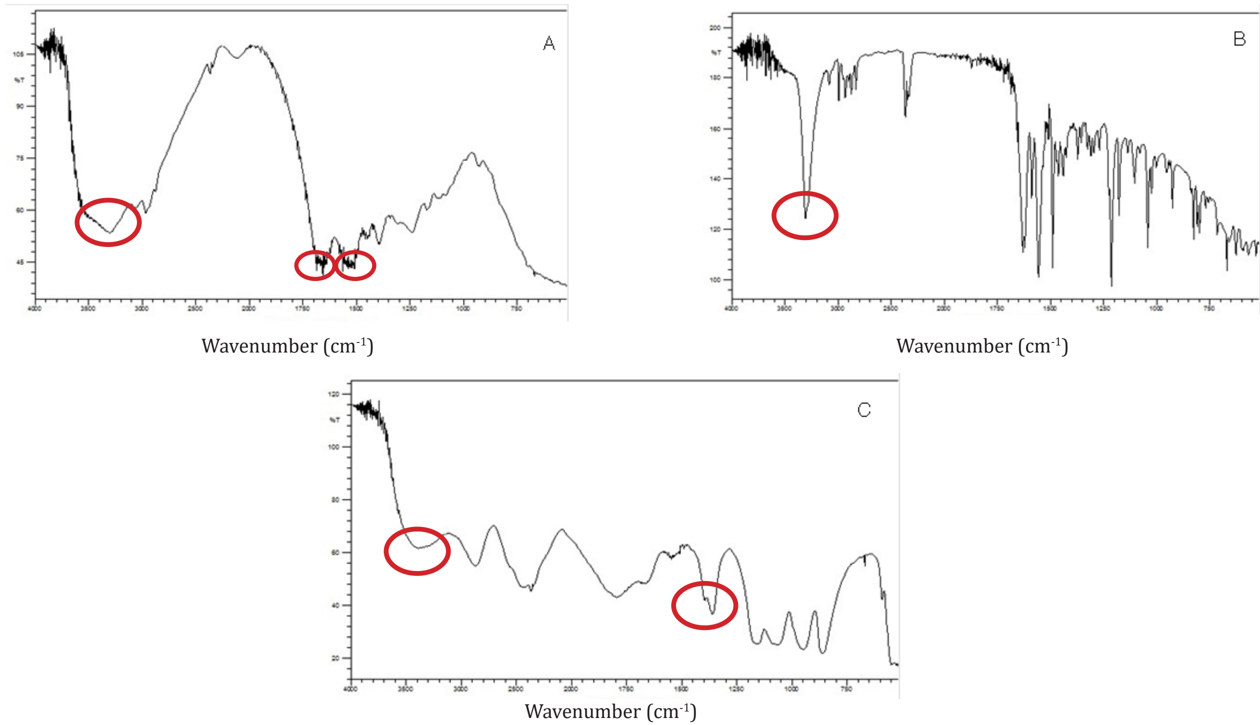

Figure 1 FTIR spectra of (A) BSA protein, (B) Melatonin and (C) Mel-BSA coupling

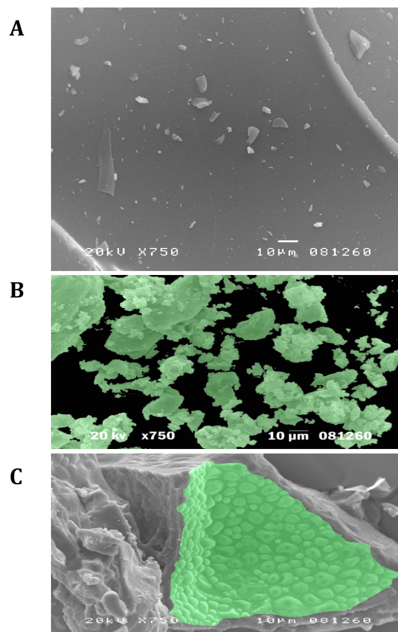

Figure 2 SEM images of (A) Bovine serum albumin, (B) Melatonin and (C) Mel-BSA. Scale bar = 5 µm

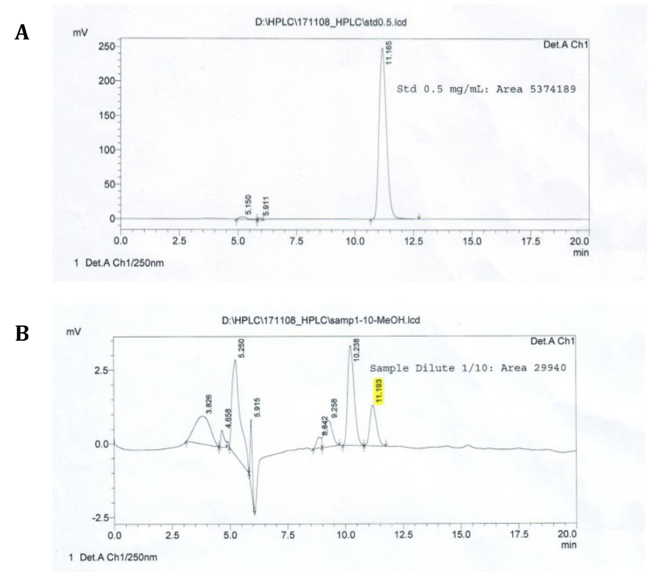

Figure 3 HPLC results of (A) Standard solution and (B) Mel-BSA

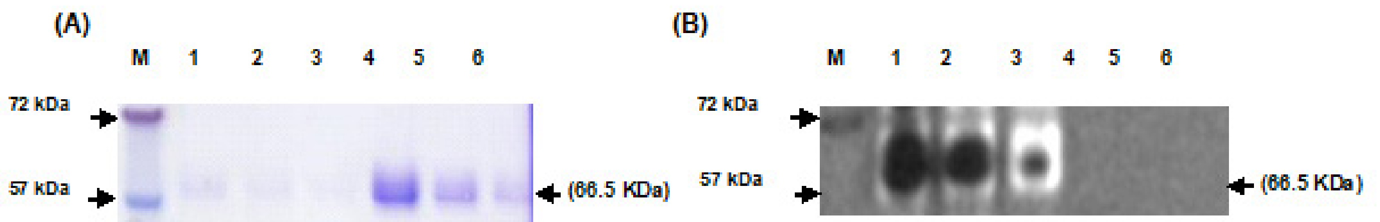

Figure 4 (A) SDS-PAGE showing Mel-BSA and BSA protein. (B) Western blot analysis showing the specificity of polyvalent antisera to melatonin after pre-absorption with 5% BSA, M= protein ladder, lane 1= Mel-BSA 0.5 µg, lane 2= Mel-BSA 0.25 µg, lane 3= Mel-BSA 0.1 µg, lane 4= BSA 0.5 µg, lane 5= BSA 0.25 µg, lane 6= BSA 0.1 µg

Enzyme-linked immunosorbent assay (ELISA)

When antisera are involved in chemical modification such as in labeling antibodies with horseradish peroxidase (HRP) enzyme, artefactual results may occur if non-purified antisera are used. Therefore, to avoid this possibility resulting from using the whole antisera in immunoassay, the immunoglobulin G (IgG) fraction of the serum was isolated using protein A/G chromatography.

Subsequent to the purification of IgG anti Mel-BSA, titers of anti Mel-BSA conjugated with HRP enzyme were determined against Mel-BSA by ELISA. Results (Figure 5) suggested that optimal dilation of anti Mel-BSA conjugated with HRP enzyme was 1:200 with detection range of 0.15-9.77 ng/mL.

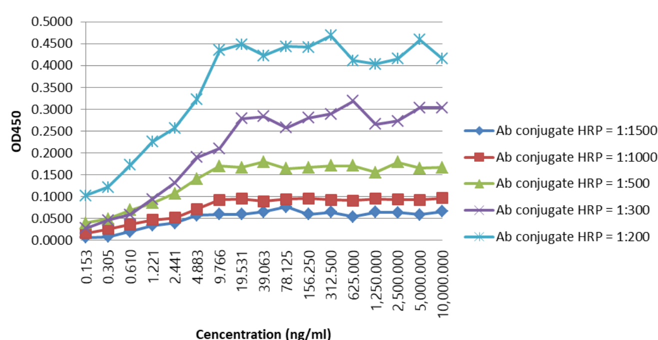

Figure 5 ELISA showing titration curves of anti Mel-BSA sera after HRP conjugation

The ELISA standard curve for estimating melatonin concentration was generated using pure melatonin at concentration 0-15 ng/mL (r^2 = 0.92).

Detection limit of ELISA using milk colloid sample was investigated by adding melatonin at concentrations of 0, 2, 5, 10, and 15 ng/mL in the milk. Results in Figure 6 showed that the corresponding melatonin concentration measured in milk sample had an identification range of 0-10 ng/mL.

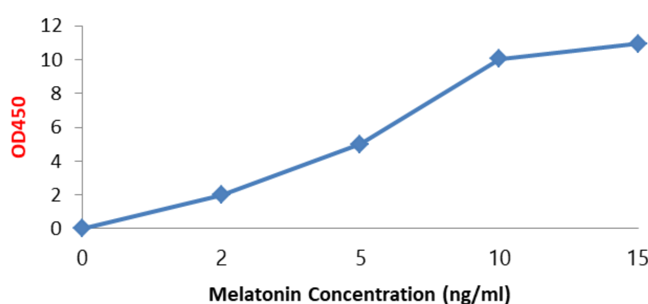

Figure 6 ELISA showing the levels of melatonin in milk

Drop test immunoassay

A drop test immunoassay was performed on various concentrations of melatonin ranging from 0.019-9.766 ng/mL. Results indicated that identification range of the drop test was similar to ELISA at 0.15-9.77 ng/mL (Figure 7 and Table 1). Additionally, when left for 1 hour to overnight there was no change in color, indicating a negative result.

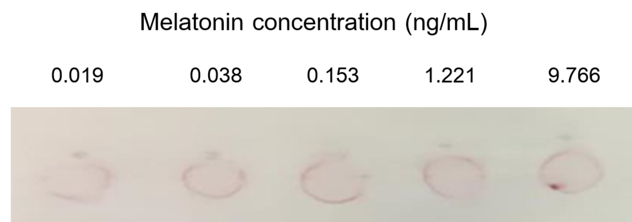

Figure 7 Drop intensity of drop test assay

Table 1 Drop intensity of melatonin concentration analyzed by ImageJ software

Melatonin concentration (ng/mL)	Average score
9.766	223
1.221	216
0.153	213
0.038	210
0.019	208

Intensity score analyzed by ImageJ software

CONCLUSIONS

A fast and reliable method for melatonin detection in all types of food samples was developed with turnaround time of no more than 20 minutes. Use of ELISA and drop test immunoassay allowed determination of melatonin concentration using simpler and less costly methods than traditional HPLC.

The material generated was highly selective for target analysis. In particular, when fabricated with nitrocellulose membrane, it showed equivalent performance with ELISA. With further development this drop test immunoassay can reduce the need to import expensive melatonin detection kits from abroad.

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