Polyclonal antibodies production from porcine gelatin and its preliminary study for immunosensor applications

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Abstract. Gelatin was extracted from porcine skin and used as an antigen to produce the polyclonal antibodies of porcine gelatin using New Zealand white rabbits for application in the development of an ELISA-based immunosensor for porcine gelatin recognition. The extraction of porcine gelatin from porcine skin was successfully performed with 20.52% of yield. The characterization performed by using UV and IR spectroscopy indicated that the purified gelatin has a comparable quality against the commercial standard of porcine gelatin. Further analysis of the gelatin with SDS-PAGE method observed five separation lines with molecular weight bands of 57.06; 49.50; 42.94; 33.89; and 29.40 KDa, indicating that the gelatin can be used as an antigen to produce the polyclonal antibodies of porcine gelatin. After five injections of gelatin into the rabbit, ELISA characterization of the purified serum of the blood rabbit showed that the antibody with a concentration of 0.289 mg/mL could be produced.

Keywords: ELISA, immunosensor, porcine gelatin, polyclonal antibody, rabbit

1. Introduction
Gelatin is a type of protein derived from natural collagen found in the skin and animal bones [1]. Commercial gelatin was generally produced from mammals, such as pork skin, bovine skin and fish [2-3]. The most widely used gelatin is the one from porcine, because of its superiority in gel strength, pressure resistance, water holding ability, and high melting point [4]. Gelatin is widely used in food products, such as candy, jelly, yoghurt, marshmallow, gelatin and pudding [5]. A practical method to analyze the gelatin content in food and non-food products is necessary due to its potency as allergens to some people as well as a requirement for halal labelling in Muslims’ community.

The analysis of porcine gelatin has been reported by some researchers using polymerase chain reaction (PCR) [6], Fourier Transform Infrared (FTIR) spectroscopy [7], reversed-phase High Performance Liquid Chromatography (HPLC) [8], and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [9]. However, these analysis methods were ineffective due to the requirements of special expertise in operation, long time analysis, as well as expensive instrumentations. Hence, a fast, cheap, effective, and easy method of porcine gelatin analysis is required. Immunosensor, such as ELISA type, is one of the methods that can fulfill the criteria. However, ELISA-based immunosensor requires antibodies as the sensing elements to recognize the antigen samples, while the antibodies can be prepared in an animal body, such as a rabbit.

The production of antibodies of porcine gelatin in rabbits for ELISA assays has been reported, involving the use of porcine and cow gelatin as the antigens to stimulate the production of the antibodies [5,10]. On the other hand, other report indicated that the porcine gelatin could be directly
produced in animal because it contains proteins with a molecular weight of more than 10 kDa [11]. Production of antibodies using direct porcine gelatin as antigen injected into white rats has been reported [12]. However, the characterization by using the dot blot method did not give good results. In this work, the porcine gelatin was directly immunized into the body of the rabbit to stimulate the production of the antibodies. As the result, specific antibodies towards porcine gelatin can be produced as determined by ELISA method.

2. Materials and methods

2.1. Ethical approval
Approval number: 083/KEH/SKE/II/2018 was obtained as a consent to perform this research and was issued by the Animal Care and Use Committee of Research and Community Services Institution, Bogor Agricultural University.

2.2. Materials
Phosphoric acid, standard porcine gelatin (G2500) and standard bovine gelatin (G6650) were purchased form Sigma Aldrich. SDS-PAGE test materials, protein marker (Thermo), phosphate buffer saline (PBS), New Zealand female white rabbits, complete Freund’s adjuvant (CFA), incomplete Freund’s adjuvant (IFA), ELISA test materials, Hi-Trap protein A column, filter paper 0.45 μm nylon membrane (Whatman) and Na.PO.

2.3. Extraction and characterization of gelatin from porcine skin
The raw porcine skin material was cleaned from the hair, meat, and fat, then cleansed under running water and arranged to be in size 2x2 cm before soaked in water and heated at 60 °C for 15 min. After cooling, it was immersed in 12 % phosphoric acid solution for 24 h. After washing, the gelatin was extracted by soaking in distilled water at 60 °C for ± 6 h. The liquid was filtered and kept at 4 °C to obtain gel form. In order to get the powder form, the gel was evaporated at 50 °C and then freezes dried. Characterization was performed by using UV-Visible and FTIR spectrophotometers.

2.4. SDS-PAGE analysis of gelatin
The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) measurements were performed using 10 mg gelatin dissolved in 2 mL PBS. Briefly, the solution was added into polyacrylamide gel consists of 14 % run gel and 4 % stacking gel. The electrophoresis was performed at an applied potential of 100 V and 50 mA per gel for 4 h using Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Following the electrophoresis process, the gel was stained with coomassie blue and washed.

2.5. Production of antibodies
Production of the antibodies of porcine gelatin was performed using New Zealand female white rabbits as the tested animal. The rabbits were injected in two routes, including intravenous and subcutaneous. Intravenous injections were performed by the injection of 0.2 mL sterile PBS containing of 10 mg gelatin into rabbits for three consecutive days. The antibodies produced in the blood serum were checked after seven days. After injections through the intravenous line, the rabbit was injected with gelatin through the subcutaneous route. This injection was performed with a mixture of 1 mL sterile PBS containing 10 mg gelatin and Freund adjuvant (1: 1). Then, blood serum was checked using Agar Gel Precipitation Test (AGPT) for antibodies on the 7- and 14- days after injection.

2.6. Monitoring of the antibodies by indirect ELISA
The microplates were coated with a gelatin antigen layer (10 mg/L, 100 μL/well) in a buffer coating (carbonate bicarbonate buffer 0.05M, pH 9.6), then covered overnight at 4 °C. After washing, the microplate was filled with the polyclonal antibody porcine gelatin antigen, incubated for 1 h, and washed. Then, 100 μL/well HRP-conjugated goat anti-rabbit IgG (1: 10,000) was added into the microplates, incubated for 1 h, and washed. The 3,3', 5,5'-tetramethylbenzidine (TMB) solution was then added into the microplates, incubated for 10 min, and added by 50 μL stop solution. The characterization was performed at the visible absorbance of 450 nm wavelengths. The microplates were washed after every coating step by using PBS Tween-20 0.05 %
2.7. Purification of the antibodies

Purification was performed using a commercial A Protein Kit (Sigma-Aldrich) to remove the polyclonal IgG antibodies from serum. Briefly, the blood serum was homogenized and filtered using a 0.45 μm nylon membrane. Two mL of the filtered serum was mixed with 4 mL solution of 20 mM Na₃PO₄ pH 7 and passed into the protein A column dropwise. The filtrate was then placed into a centrifuge tube (containing 0.2 mL buffer tris-HCl pH 9) to 1 mL/fraction. Each fraction was characterized by UV spectrophotometer at a 280 nm to calculate the concentration.

3. Results and discussion

3.1. Characterization of gelatin from porcine skin by using UV-Vis and IR spectrophotometer

The extraction of porcine gelatin was carried out by cleaning and soaking the porcine skin with warm water at around ± 60 °C. After the skin became soft, the skin was immersed in acid to convert a triple-helix collagen fiber into a single chain. The acid also served in the demineralization process to remove the minerals, such as calcium salts, due to the swelling of the materials. The last stage of the extraction process was heating, as the gelatin dissolves in warm water (T ≥ 40 ºC). The effectiveness of the gelatin-preparation method was confirmed with the total yield of 20.52 %.

Comparison of the UV-Vis spectra of the extracted and standard commercial porcine gelatin is shown in figure 1. The amino acids in gelatin can be identified using the UV spectrum at the 210–240 nm which shows the absorption of the chromophore groups, including glycine, proline and arginine amino acids.

Further characterization was performed using FTIR to determine the functional groups in the gelatin. Gelatin normally consists of hydroxyl groups (O-H), carbonyl groups (C=O), and amine groups (N-H). Figure 2 shows a wide absorption band at 3100–3500 cm⁻¹, which confirmed the presence of the stretching vibration bands of O-H as well as N-H groups. The presence of O-H groups in gelatin might be also due to the use of water in the extraction process. In addition, the absorption at 2924 cm⁻¹ indicated C-H stretching vibration, while three different absorption peaks at 1627 cm⁻¹, 1523 cm⁻¹, and 1235 cm⁻¹ indicated the presence of the groups of amide I, amide II, and amide III, respectively.

3.2. Protein analysis in the extracted porcine gelatin using SDS PAGE

Analysis of the gelatin was also performed using SDS PAGE method by separating the protein according to the molecular weight. Comparison with the standard commercial porcine gelatin and bovine gelatin was conducted to determine the difference in protein compositions. Figure 3 shows the different patterns of SDS PAGE results of bovine and porcine gelatin. Gelatin from porcine, including the extracted one, typically generated five protein bands, indicated that the gelatin composed of five main proteins. Whereas, the standard porcine gelatin shows molecular weight bands at 59.8250; 51.9017; 45.0278; 36.5341; and 29.4020 KDa, the isolated one shows the bands at 57.0579; 49.5011; 42.9451; 33.8905; and 29.4020 KDa. On the contrary, the standard bovine gelatin generated only one
Figure 2. FTIR spectra of the extracted porcine gelatin (red line) in comparison with the commercial one (black line) from Sigma-Aldrich

Figure 3. SDS PAGE bands of (a) the commercial and (b) the extracted porcine gelatin in comparison with (c) the commercial bovine gelatin

band at molecular weight of 47.2115 KDa. The results suggested that the porcine gelatin protein can fulfill the criteria as antigen to produce the antibody, as previously reported that protein substance must have a minimum molecular weight of 10 KDa to be effective as immunogen [11]. Moreover, the different protein compositions between bovine and porcine gelatin indicated the possibility to distinguish the gelatin from bovine and porcine.

3.3. Production of antibodies and characterization by using ELISA
The polyclonal antibodies were produced by the immunization of porcine gelatin in the New Zealand female white rabbit as the tested animal. Initially, the rabbit was immunized through intravenous, then through the subcutaneous. The first injection was performed two times to observe the rabbit’s response to the gelatin antigen, while the subcutaneous injection was aimed to promote the immune responses. The last injection was repeated three times by mixing the antigen with Freund’s adjuvants to induce the immune systems for long-term injections.
Table 1. Absorption of TMB during ELISA measurements of the rabbit blood serum during porcine gelatin injection

| Serum day to porcine gelatin extraction | Absorption of porcine gelatin extraction |
|----------------------------------------|-----------------------------------------|
| 0                                      | 0.165                                   |
| 10                                     | 0.906                                   |
| 14                                     | 0.870                                   |
| 18                                     | 0.514                                   |
| 21                                     | 0.653                                   |
| 31                                     | 0.395                                   |
| 38                                     | 1.313                                   |
| 46                                     | 1.336                                   |
| 52                                     | 1.242                                   |
| 59                                     | 1.320                                   |
| 65                                     | 1.328                                   |

Monitoring of the presence of antibodies was performed with analysis of the blood serum during the injection by using ELISA method. The produced antibody was used as the sensing materials, while porcine gelatin was used as the sample and TMB as the label. The amount of TMB is equivalent to the amount of the antibody. Table 1 shows the visible absorption of the blood serum from the initial stage of injection. The table shows that the absorbance has a tendency to increase and be saturated at around the 38th day. The results suggested that the rabbit has responded to the porcine gelatin antigen by producing antibodies.

The purification of the produced antibodies was performed by using Protein A filtration on the 65th days serum after totally 5 times injections. The results of the purification showed the IgG concentration of the antibody was 0.289 mg/mL.

4. Conclusions
Gelatin can be extracted from raw material of porcine skin with acid treatment with the yield up to 20.52%. Analysis with SDS PAGE showed that the porcine gelatin has five separated bands with the molecular weights of more than 10 kDa. Injection of the extracted gelatin to rabbit Polyclonal antigens of the porcine gelatin can be produced in the rabbit after 65 days with an IgG concentration of 0.289 mg/mL.

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