International Journal of Progressive Sciences and Technologies (IJPSAT)
ISSN: 2509-0119.

Vol. 44 No. 2 May 2024, pp. 189-193

Immunogenicity Of Boiled Pork Extracts And The Protein Band From SDSPAGE Of Fresh Pork Extracts

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***Abstract*— The aim of this study was to analyze the immunogenicity of boiled pork extract antigens and the isolate SDSPAGE protein bands of fresh pork. The antigens were injected subcutaneously, repeatedly at two-week intervals into two local rabbits. Antibodies were isolated two weeks after the last booster. Analysis of the immune response was carried out using the indirect immunoblot assay. The results of this study showed that boiled pork antigens were more immunogenic than SDSPAGE bands of fresh pork. The specific activity of the antibodies obtained was about six times higher than the antibodies from rabbits vaccinated with SDSPAGE bands of fresh pork extract. Whether the antibodies obtained can be used to develop meat adulteration authentication test kits still needs to be investigated.**

Keywords— Antibody; Boiled Pork Extracts; Immunoblot; Meat Adulteration; SDSPAGE Bands.

I. INTRODUCTION

The aim of this study was to analyze the immunogenicity of two types of antigens, boiled pork extract and protein bands isolated from sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDSPAGE) gels of fresh pork extract. Understanding the immunogenicity is important in terms of producing antibodies that can be utilized in the development of immunodiagnostics of food contamination due to the mixing of meat from various animal species, including pork, which is often carried out by irresponsible persons, for economic reasons [1], [2].

The contamination of pork or its derivatives in a food product is not desired by some consumers due to certain considerations, such as religious, cultural, and public health concerns. For this reason, various methods have been developed to authenticate that a food product is completely free from being contaminated with pork components and their derivatives, ranging from physicochemical reaction-based methods, immunological- based assays such as ELISA, up to molecular biology level methods [3], [4], [5]. The advantages and disadvantages of each method have often been reviewed, such as by Li et al. [1].

Immunoassay-based methods are commonly used because the methods are practical and have been widely developed [6]. This method requires antibodies that are specific to pork components. To produce specific antibodies, a typical immunogen is required [7], [8]. In relation to the development of kits to authenticate that food is free from meat adulterations, specific antibodies that can detect denatured proteins because of food processing play important roles [9], [10], [11]. Depamede [12] successfully developed a lateral flow immuno-rapid test to detect pork components in processed food, but the kit's capability is only for non-denatured food products. To overcome these limitations, several efforts have been made [13] but the results are still not optimal.

Some studies have shown that antibodies to denatured pig components, such as antibodies to denatured pig blood, have been

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successfully produced [14], whereas the production of antibodies to denatured pork extracts is still limited. For developing immunoassays, antigen specificity and immunogenicity play an important role in generating the required specific antibodies. Several studies reported that antigens derived from SDSPAGE gel protein bands were immunogenic and able to produce specific antibodies [15], [16], [17]. In this study we report the immunogenicity of two types of antigens, the boiled pork extract and the protein bands of fresh pork extract components that have been separated using SDSPAGE.

II. Materials and Methods

2.1. Vaccine preparation

In this study, the antigen used was derived from pork, purchased at a traditional wet market in Mataram city, Lombok, West Nusa Tenggara, Indonesia. The pork was extracted by a modified method from Nurhaerani et al. [13]. One gram of meat was blended and homogenized in 3 ml of 0.9% NaCl solution then centrifuged at 4°C, 4500rpm for 30 minutes. The pellet was collected, resuspended, and homogenized in 0.9% NaCl solution then centrifuged at 4°C, 4500rpm for 30 minutes. The supernatant was collected, referred to as fresh pork extract, and then divided into two parts. One part was used for vaccine preparation through SDSPAGE separation process while the other part was used for vaccine preparation through boiling.

The first vaccine (Vaccine A, boiled pork extract) was prepared by boiling fresh pork extract at 100°C for 15 minutes. After cooling, it was centrifuged for 10 minutes at 4°C, the supernatant was collected and stored frozen until used for the vaccination process. The second vaccine (Vaccine B) was a protein band of fresh pork extract isolated from 10% SDSPAGE. The SDSPAGE was done according to Laemli (1970) [18] modified by Nurhaerani et al. [13]. Target protein bands specific to the pig species were excised with a blade, crushed, and stored frozen until used for the vaccination process. Protein concentration was determined using a spectrometer (NanoDrop) at OD 280.

2.2. Rabbit vaccination

In this study, two local adult male rabbits in healthy condition were used. The use of experimental animals in this study is in accordance with the provisions of animal ethics that apply at the Faculty of Animal Husbandry, University of Mataram, No. 361/UN18.F7/ETIK/2023. Vaccination using emulsion vaccine, a mixture of complete Freund's adjuvant and vaccine (1:1), was carried out based on Kisworo and Depamede [19] and [13] with several modifications. Vaccine doses for the first vaccination and booster were equivalent to 200 ug and 100 ug of protein per rabbit respectively and administered subcutaneously. Boosters were carried out three times with an interval of two weeks. Blood samples were collected weekly via the earlobe vein, then two weeks after the last booster the blood was harvested from the heart. Before harvesting, each rabbit was anesthetized with ketamine HCl combined with xylazine as modified from [20].

2.3. Purification and analysis of specific activity of antibodies

Isolation and purification of antibodies were carried out in two stages, namely salt precipitation using 50% ammonium sulfate then followed by a Protein A affinity column based on [19] and [13] with minor modifications.

Specificity activity of antibodies was analyzed using an indirect immunoblot assay based on Wariata et al. [21] with slight modifications. The antigen to be tested in the form of pork extract is dropped at 10 ug per spot on the teeth of the comb shaped solid support. After drying, they were blocked using 4% skim milk in Tris buffered saline containing 0.05% Tween 20 (TBST) for 60 minutes at 37°C then washed three times using TBST. After washing, the combs were soaked in the antibodies appropriate to the treatment target with a concentration of 10 ug per tooth comb, for 60 minutes at 37°C. After incubation and washing the comb was soaked in the alkaline phosphatase conjugate secondary antibody (goat antirabbit IgG) according to the manufacturer's instructions (Sigma, A-3937), for 60 minutes at 37°C. After 3 washings using TBST, the comb was soaked using NBT/BCIP solution (Roche, Germany) in a dark room until a blue-black spot formed. The reaction was stopped using pure dH2O according to the manufacturer's instructions. The intensity of the color formed was quantified using the ImageJ 1.54i software tool (http://imagej.org). Visualization of antibody purity levels was carried out using SDSPAGE 10% based on the Lameli [18] method modified by [22] and [13].

III. Results and Discussion

The main objective of this study was to analyze the immunogenicity of denatured pork antigens. Antigens including boiled pork extracts and SDSPAGE pork extracts protein bands were inoculated into local rabbits. Post vaccination antibodies from each

treatment were purified and analyzed using immunoblot as presented in Figure 1.

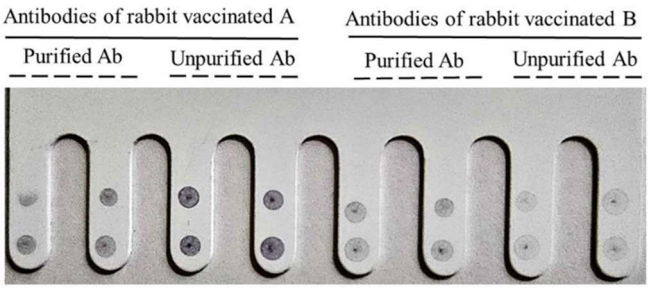

Fig. 1. Immunoblot analysis using antibodies from rabbits vaccinated with boiled pork extract (A) and SDS-PAGE protein bands of pork extract (B). Each antibody consists of purified and unpurified antibodies

Figure 1 shows clearly that antibodies produced by vaccination using boiled pork extract are generally stronger than antibodies from rabbits immunized using SDSPAGE protein bands of pork extracts. The difference was clearly seen in both purified and unpurified antibodies. Qualitatively, the immunogenicity of the 15 min boiled pork extract antigen was more immunogenic than that isolated from the SDSPAGE bands. Immunogenicity of boiled pork has been reported by [13] which similar to the results of this study. Chen and Hsieh [23] also reported their success in producing monoclonal antibodies against heated porcine muscle, even at autoclave temperatures.

The use of antigens isolated from SDSPAGE bands as vaccines was reported by several researchers [15], [16], [24], however our results are different from those previously reported. In this study, the immunogenicity indicated by the proteins produced from the SDSPAGE bands of pork extract was not as strong as the boiled pork antigen.

To analyze the results of the purification process of the antibodies quantitatively, we performed dot blot analysis using the ImageJ 1.54i software application (http://imagej.org). The analysis results are presented in Table 1. From the aspect of specific activity, the antibodies obtained by vaccination using boiled pork are about six times that of antibodies from rabbits vaccinated with SDSPAGE sliced bands of pork extract.

TABLE I. SPECIFIC ACTIVITY OF PURIFIED ANTIBODY AGAINST PORK EXTRACTS

Purification step	**Total volume (ml)**	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Unpurified Ab A (a)	5	250.00	30.79	0.12	1.00	100.00
Purified Ab A (a)	7	1.98	22.19	11.20	90.97	72.08
Unpurified Ab B (b)	5	241.10	10.73	0.04	1.00	100.00
Purified Ab B (b)	6	7.80	14.82	1.90	42.69	138.12

(a) Rabbit vaccinated with antigens of boiled extract pork

(b) Rabbit vaccinated with SDSPAGE sliced bands of pork extract

Further attention was then focused on the antibodies obtained from rabbits immunized with boiled pork extract antigens. The purity level of antibodies was analyzed using 10% SDSPAGE and presented in Figure 2. It appears that antibodies with high purity level were obtained after purification using protein A column. When these results are combined with the results of immunoblot analysis (Figure 1 and Table 1), it appears that antibodies obtained from rabbits vaccinated with boiled pork extract and purified using a combination method between 50% ammonium sulfate precipitation and protein A column have the potential to support the development of immunodiagnostics of processed food adulteration.

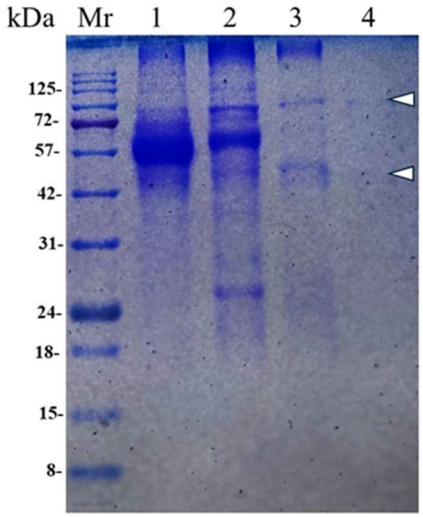

Fig. 2. Representative of a 10% SDSPAGE of unpurified and purified antibodies from local rabbits vaccinated using boiled pork extract antigens. Arrows indicate denatured IgG; molecular weight (Mr) standard (BLUelf Prestained Protein Ladder, GeneDirex Inc); unpurified Ab (lane 1); 50% ammonium sulfate precipitate (lane 2); protein A column fraction of purified Ab (lane 3-4)

IV. Conclusion and Suggestion

The results of this study showed that boiled pork extract was more immunogenic than SDSPAGE bands of pork extract. The specific activity of the antibodies produced was about six times higher that of antibodies from rabbits vaccinated with SDSPAGE bands of pork extract. The antibodies obtained still need to be further investigated, whether they can be used for the development of a meat adulteration authentication test kit.

V. Acknowledgment

This study was funded by the Institute for Research and Community Engagement, University of Mataram, DIPA BLU PGB Scheme No. 2590/UN18.L1/PP/2023. We are grateful to Mr. Dedi Isnaini at Microbiology and Biotechnology Laboratory, and to Mr. Khalid at the Immunobiology Laboratory, Unram for their technical assistance. The combs for immunoblot were the generosity of Prof. Mulyanto, WNT Hepatitis Laboratory, Mataram, Indonesia.

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