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POLYCLONAL ANTIBODIES AGAINST "PROTEOLIPID" OF PORK (*SUS SCROFA/SUS VITATUS*) EXTRACTED BY ETHER-TRICHLORO ACETIC ACID (TCA) - ACETONE METHODS

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ABSTRACT

In Indonesia, the practice of mixing pork with beef, goat, lamb or chicken meat is still often done by irresponsible people. This study aims to produce polyclonal antibodies against proteolipid pork, as a raw material for the development of immunodiagnostic against pork contaminated products. In this study proteolipid vaccine were isolated from local pork through a combination method of ether and trichloro acetic acid extractions. Polyclonal antibodies were produced using a local rabbit and three local Balb/c mice, according to standard immunization procedures. Immunodotblot results showed the antibody produced were able to react positively to the extract of raw pork as well as cooked pork. Antibodies produced in rabbit, but not in mice, still showed a cross reaction against beef extracts. Whether these antibodies can be used for the development of diagnostics against pork contamination, the related studies are being undertaken in our lab.

Keywords: Meat adulteration, pork, lateral flow assay, immunodotblot.

1. INTRODUCTION

For some people or communities, the presence of pork or components of pork in food, cosmetics, or pharmaceuticals is something that must be avoided or even prohibited. This is due either to religious concerns- or to health reasons, such as allergy problems (Asero et al. 1997, Atanaskovic-Markovic et al., 2007). However, in Indonesia there are still many irresponsible people who conduct defrauding or adulteration, by mixing pork or pork components in their products without providing adequate information or undeclared ingredients. This can deceive or even harm the consumers. For that we need to develop a practical tool or kits that enable us to detect pork or pork components in such products.

Several detection methods have been developed, including electrophoresis techniques (Kim and Shelef, 1986), isoelectric focusing (Jaussen et al., 1990), chromatography (Saeed et al., 1989), enzyme-linked immunosorbent assay (ELISA) (Hsieh et al 1996, Chen et al. 1998, Hsieh et al. 2002), DNA hybridization (Chikuni et al. 1990, Ebbehøj, 1991), and polymerase chain reaction, PCR (Matsunaga et al., 1990) or polymerase chain reaction-restriction fragment length polymorphism, PCR-RFLP (Mutalib et al. 2002). Furthermore, researchers from Malaysia, Aida et al. (2005) reported their results with a PCR method to identify the presence of meat and lard components based on the conserved region of cytocrome b gene (cyt b) mitochondria of porcine. Identification of pork based on DNA technology delivers results with high accuracy and sensitivity. However, this method as well as some of the methods mentioned above require rather sophisticated equipment and trained personnel to do it, otherwise it takes an average time of over two or three hours at a relatively high cost, e.g. 750,000 IDR or about 54 USD per test at the referral laboratories.

Several years ago, we have conducted a preliminary research on the development of rapid immunochromatography tests to detect pork components mixed in beef or chicken meat (Depamede, 2011). The detection capability of the kit was up to 1 kg of pork per 5-10 tons of raw beef or chicken meat. The test 'sensitivity' level of 1/5,000 ratios for the field condition is sufficient, given the fact that most of the irresponsible producers generally mix the meat with the ratio of pork and non-pork components of around 1: 2 to 5 parts. This is based on the producer's calculations to benefit from the mixing without being able to be noticed by the consumers in laity or with the naked eye.

The rapid detection kit we developed used the principle of immunochromatography combined with antigen-antibody reactions. The problem we encountered with the kit was that our kit could not detect any fat or proteolipid components of pork or pig. This was probably due to the antibodies we used were antibodies specific to pig-IgG, not against fat or proteolipid of pigs.

For the development of IC kits on proteolipid porcines, antibodies against fat or proteolipid of pigs are required. For that we have conducted a study on the production of polyclonal antibody against "proteolipid" of pork (Sus Scrofa/Sus Vitatus) that had been extracted by means of combination of trichloro acetic acid (TCA) – acetone methods.

2. MATERIALS AND METHODS

2.1. Materials

Materials used in this study included chemicals and biological materials. Chemicals consisted of saline salt solution, Freund's complete adjuvant, phosphate buffered saline (PBS), tris HCl-Trizma, bovine serum albumin, reagents for immunoblot assay, Anti-rabbit IgG (whole molecule)–Alkaline phosphatase secondary antibody, BCIP-NBT solution kit, chilled of trichloro acetic acid (TCA) and acetone, ammonium sulfate. Biological materials consisted of pork marbling, as vaccine-making materials, which were bought from wet markets in Mataram City, Lombok. Three female adult Balb/c mice and a male local rabbit were used for immunization treatments. For assessing of the antibody produced, extracts of meat and fat from chicken, beef, and porkpurchased from the wet markets were applied.

2.2. Technical equipment and equipment

Microtubes, 1 and 3 ml syringes, safety gloves, safety masks, analytic balance, mortar crusher, oven, microwave, water bath, refrigerated high speed centrifuge, apparatus for immunoblot.

2.3.Methods

Vaccine preparation was carried out based on Hsieh and Gajewski(2016) and Bensadoun et al. (1974) with modifications. The adipose tissue was slashed out from parts of the meat or muscle tissue. After freezing all of the fat parts from the muscle tissue, the adipose tissue was then finely sliced and weighed as much as 50 g. The 50 g of tissue was placed in a beaker glass or mortar, and then heated to 195°C until melted. Immediately after heating, when the fat was still melted, the liquid fat was poured off and the crude components were left in the beaker glass or mortar. The crude components were immersed in PBS (1 gram tissue in 3 ml PBS) and blended with a mortar, then centrifuged at 3000 rpm at room temperature for 5 minutes. Following this, 3 portions of aqueous part (the supernatant) were mixed with 1 portion of saturated TCA (25% v/v TCA) for one hour, and then the mixture was centrifuged at 12,000 rpm, 5 minutes at room temperature. The pellets obtained were washed three times with chilled acetone, by centrifugation at 12,000 rpm, 1 minute at room temperature. Finally, the pellet was dissolved in complete Freund's adjuvant and used for immunization process.

Immunization processes were carried out according to Leenaars and Hendriksen (2005) on 3 adult local Balb/c mice and 1 adult local rabbit. After three times immunizations, with 2 weeks interval, the animals were sacrificed according to the procedure in our laboratories and the blood was collected. Antibodies were isolated from the sera by means of ammonium precipitation standard method (Wingfield 2001). The purified antibodies obtained from mice or rabbit were tested for their immunogenicity by means of immunodotblot assay against the extracts of pork, chicken, and beef. Data obtained in this study were analyzed descriptively

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Vaccine preparations

Figure 1 is a representation of the results of a step during the vaccine preparation process. Cloudy fluid (arrow) appeared after the addition of 25% (v/v) of TCA. This suggests the presence of protein in the adipose component of pork used in this study. Furthermore, the precipitate obtained after centrifugation and washing using cold acetone was taken and used as a vaccine

Figure 1. A representation result of a step during the vaccine preparation process. Cloudy fluid (arrow) indicated the presence of "lipoprotein" after the addition of 25% TCA.

3.1.2 Antibody

Antibodies obtained in this study were semi purified using ammonium sulfate precipitation method. The representation of calculation approach of the purity and activity of the antibody is presented in Table 1.

Table 1. Representation of calculation the purity of proteolipid antibodies (Ab) produced in rabbit, post-purification using ammonium sulfate precipitation method

No.	Component	Vol (µl)	Protein OD280 (µg/µl)	Total protein (µg)	Specificity (U/µg)	Total activity (U)	Fold of purity	Recovery (%)
1.	Serum containing antibodies	500	6	3000	6	18000	1	100
2.	Purified Antibody	100	1.4	140	100	14000	16.7	77.8

3.1.3 Immunodotblot analysis

Immunodotblot assay was used to analyze the activity and specificity of antibodies produced in this study. Figure 2.A, and 2.B are representations' of antibodies against pork "proteolipid" produced in rabbit and Balb/c mice respectively.

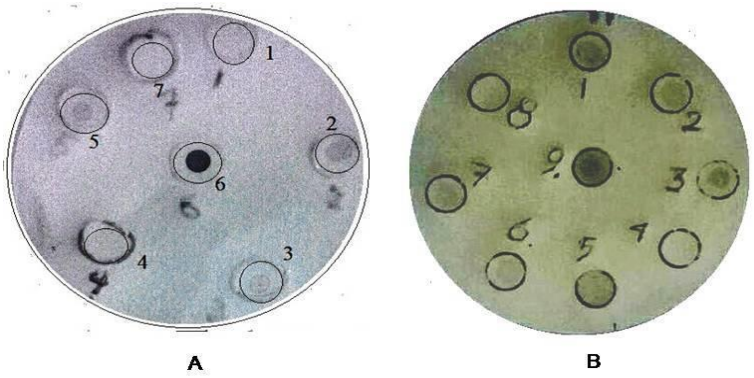

Figure 2. Representations of immunodotblot results of pork proteolipid anti body produced in Rabbit (A) or in Balb/c mice (B).

Figure 2.A. Antibody reactions against 1 and 7 vaccine, 2 extract of cooked pork, 3 extract of raw pork, 4 extract of cooked chicken, 5 raw beef, and 6 normal rabbit serum.
Figure 2.B. 1 and 2 vaccine, 3 extract of raw pork, 4 extract of raw beef, 5 extract of raw chicken, 6 extract of raw mutton, 7 extract of cooked beef, 8 extract of cooked chicken, and 9 positive control.

3.2 Discussion

This study aims to produce antibodies against pork "proteolipid", which will then be used to develop a meat and pork fat detection kit. For this purpose, antigen from adipose pork tissue was isolated to be a vaccine material. Isolation of adipose has been done elsewhere, such as by Hsieh and Gajeweski (2016). However, the production of antibodies against adipose pork has not been widely reported. Our preliminary study to produce antibodies against adipose pork has not yielded optimum results, when the antigen was isolated on the ether based method. This was in contrast to the report of Kim et al. (2017) who claimed to have successfully made monoclonal antibody that specific to pork fat protein without cross-reaction to other animal meats and fats. Hsieh and Gajeweski (2016) have also succeeded in extraction the immunogenic adipose components by modifying the ether-based extraction method. Faced with the difficulties that we had experienced in the preliminary study, we then changed the antigen isolation strategy by implying precipitation techniques using modified TCA-acetone extraction combining with the method has been used by Hsieh and Gajeweski (2016).

Immunization using "proteolipid" isolated by the modified TCA-acetone method, showed positive results as presented in Figure 2.A and 2.B. The TCA-acetone method has been long-established as a conventional method, including for lipoproteins extraction such as reported by Bensadoun et al. (1974). Up to now this method is still used and modified for several purposes (Hao et al., 2015, Vincent et al., 2016). In this study the antibodies produced in rabbits reacted to raw pork extract, as well as to the extract of boiled or cooked pork when assayed using the immunodotblot method (Fig. 2.A). However, the antibody still gave cross reaction to raw beef extract. This is probably because the antibody is a polyclonal antibody and was of a semi purified one, i.e. based on the ammonium sulfate precipitation alone. Kim et al. (2017) reported that their monoclonal antibodies did not cross-react with other meat components when tested using either ELISA or westernblot techniques. Interestingly, the antibodies we produced on Balb/c were more sensitive and more specific than those produced in rabbits (Fig. 2.B). The problem is that the quantity of polyclonal antibodies obtained from mice is so limited hence it is difficult to be used for diagnostic development. Depamede (2011) successfully developed lateral flow immunodiagnostic to detect pork component in a ratio of one part of raw pork in 5000 parts of either raw beef or chicken. The problem with the kit was that it was only capable to detect raw pork instead of cooked pork. The polyclonal antibodies we obtained in this study showed the potential reaction to the extract of cooked pork. Whether the antibodies are able to be used for lateral flow immunoassay, further study needs to be done. Currently the study is under construction in our lab.

4. CONCLUSION

Antibodies against proteolipid isolated from local pork have been produced successfully in rabbit and mice. Immunodotblot results showed the antibodies produced were able to react positively to the extract of raw pork as well as cooked pork. Cross reactions to beef extracts were still observed in antibodies obtained from rabbit, while similar things were not found in antibodies produced from mice. Studies on the immunodiagnostic development to evaluate whether these antibodies can be used for the development of diagnostics against pork contamination are being undertaken in our lab.

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