

Reducing fat deposition by immunisation against adipocyte membranes in poultry. I. Antigen production, antibody development and characterisation

(Mengurangkan lemak melalui imunisasi terhadap membran adiposit pada poltri.

I. Pengeluaran antigen, pembentukan antibodi dan penciriannya)

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Key words: poultry, fat reduction, immunisation, adipocyte membranes, antibodies

Abstrak

Sel lemak daripada ayam telah berjaya diasingkan dengan menggunakan kaedah yang diubahsuai daripada kaedah yang digunakan untuk tikus. Jumlah sel lemak yang didapati dari depo lemak di bawah kulit sahaja lebih banyak ($p < 0.05$) berbanding dengan gabungan lemak dari depo lemak di bawah kulit dengan depo lemak abdomen. Depo-depo ini mengandungi kepekatan protein yang sama (purata sukatan $1.05 \pm 0.22 \mu\text{g}/\mu\text{L}$). Suntikan $250 \mu\text{g}$ protein ini kepada ayam belanda mengakibatkan pengeluaran antibodi yang tinggi (kecairan 1:100 000). Kajian ciri-ciri antibodi ini menunjukkan bahawa ia bertindak balas secara khusus dengan 9 jenis protein daripada membran sel lemak. Antibodi ini juga boleh bertindak balas dengan membran organ lain seperti jantung, buah pinggang, otak, paru-paru dan, otot, tetapi tidak bertindak balas dengan membran hati atau darah. Bukti reaksi ini selanjutnya disokong oleh kajian cerakinan kesitotoksikan. Dalam kajian ini didapati antibodi bersifat sitotoksik terhadap membran sel lemak (2 kali ganda sitotoksiti serum biasa) tetapi tiada kesan sitotoksiti terhadap membran sel darah merah. Oleh itu antibodi yang dikeluarkan daripada sel lemak mempunyai potensi untuk memusnahkan membran sel lemak tanpa kesan sampingan yang ketara terhadap ayam yang disuntik.

Abstract

Chicken adipocytes were successfully extracted using a modified protocol to that for rat tissues. The yield of adipocytes from the subcutaneous depo alone was significantly ($p < 0.05$) higher than depositions from the subcutaneous plus the abdominal region with an almost similar amount of protein (average at $1.05 \pm 0.22 \mu\text{g}/\mu\text{L}$). An injection of $250 \mu\text{g}$ of these proteins elicited high antibody production in turkeys (dilution of 1:100 000). Characterisation of these antibodies showed that they react specifically with 9 proteins from the adipocyte membranes and also other proteins from the membranes of other organs including the heart, kidney, brain, lung and muscles, but not from those of the liver or the blood. This evidence was further supported by cytotoxicity assays where the antibodies were cytotoxic to the adipocyte membranes (double to that of the normal serum), but not to the red blood cell membranes. The antibodies therefore, have good potential in destroying fat cell membranes without obvious side effects to the chicken.

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Introduction

Excessive fat deposition in poultry presents big problems in terms of wasting feed energy as wasted fat tissues and the production of fatty and unhealthy poultry meat. In the Malaysian context, it also creates a further problem of becoming an environmental pollutant in the disposal systems of poultry processing plants where it clogs waterways and delays fermentation processes in the fermentation tanks. Many approaches have been taken by poultry producers to reduce excessive fat deposition in poultry. The new method of manipulating carcass fat in animals by an immunological approach has been tried on different species (Butterwith et al. 1989; Flint 1990 Nassar and Hu 1991 and Kestin et al. 1993). Using this approach in poultry Butterwith et al. (1989) found that there was good antibody production in sheep immunised with chicken adipocyte membranes, but these sheep antibodies were relatively non specific to chicken adipocyte membranes. The antiserum produced had considerable reactivity towards other cell types including liver and red blood cells. It could be that sheep; a more unrelated species to chicken produced antibodies that cross-reacted more with the other membranes. In this study therefore, the antibodies were produced in turkeys instead of sheep since turkeys are more related to chicken than sheep.

Materials and methods

Antigen preparation

Preparation of adipocyte membranes About 50 g of fat tissues was removed from the subcutaneous and abdominal region of commercial spent layer chicken. Tissues were minced in physiological saline (0.9%) at 37 °C and individual adipocytes were isolated by colleganese digestion (Belsham et al. 1980). The membranes of these adipocytes were extracted following procedures that had been modified from the basic rat procedure of Flint et al. (1986). About 4 g of the isolated adipocytes were pooled from the different depos and later

homogenised in 2 or 3 volumes of membrane extraction media (MEM) containing 1 µL 0.2M phenylmethyl sulfonyl fluoride (PMSF) and 1 mg/mL of Bacitracin. The homogenate was then spinned at 3 000 rpm at 27 °C for 7 min. After removing the top solidified fat layer, the remaining portion was centrifuged at 25 000 rpm at 4 °C for 1 h. The pellet obtained was resuspended in 32% sucrose and homogenised in ground glass homogeniser. The homogenate was layered with MEM without PMSF onto 32% sucrose in a centrifuge tube and spun at 20 000 rpm for 1 h at 4 °C. The membranes were harvested from the top interface of the sucrose layer and diluted with phosphate buffered saline (PBS) before spinning again at 20 000 rpm for 1 h. The membrane pellet obtained was resuspended in 2–5 mL of PBS for the determination of its protein content (Bradford et al. 1976).

Antibody development

Immunisation About 250 µg of the adipocyte membranes was injected into the neck areas of 10 male turkeys. Immunisations were done at monthly intervals for 5 months. Blood was collected 2 days after every immunisation and the serum was used for analysing antibody titre. Antibody assessment used the standard ELISA sandwich system where the second antibody was conjugated to the enzyme phosphatase. The optical densities of the colour developed were read using the ELISA reader (Dyna Tech. Ltd.) and readings were averaged for each bird and for positive birds.

Antibody testing (specificity)

Preparation of other membranes The extraction of membranes from the heart, kidney, brain, liver, spleen, blood, lungs and muscles was basically similar to that of the adipocytes membrane extraction except at certain stages. During the extraction of membranes from the organs, homogenising was done cold (4 or 0 °C). The initial ultra centrifugation was done for 2 h instead of 1 h

as in the adipocytes. The final membranes of the organs were stored in diffuse suspensions instead of in a pelleted form. The quantity of proteins present in these extracted membranes were assessed using the Bradford (1976) method. The proteins were then used for the analyses.

SDS-PAGE and Western blotting SDS-PAGE was performed by the method of Laemmli (1970). Gels were blotted onto immobilon PVDF transfer membrane (Millipore) using a Bio-Rad. Trans-Blot Electrophoretic Transfer Cell. A continuous buffer system was used for the transfer of media mixture which contained 25 mM tris (pH 8.3), 192 mM glycine and 20% methanol. Transfer time was overnightly with 50 volt and 25 Amp current using programmable power pack. Standard field and plate electrodes were used to transfer the current. Chromogenic Western Blotting Kit (Boehringer Mannheim) did membrane blocking for non-specific binding sites. Blots were then washed 3 times with washing buffer and incubated overnight with primary antibodies of 1 000-fold dilution of antiserum in the same buffer. Membranes were washed 4 times for 10 min each in TBST (0.1% Tween 20 in tris buffered saline, pH 7.5). Bound antibodies were detected using a ready to use substrate from the same kit. The second antibody however was prepared from goat anti-turkey IgG conjugated with alkaline phosphatase. Membranes were then dried and molecular weight calculated by comparison with prestained protein marker standard (Bio-Rad) run on the same blot.

Antibody testing (cytotoxicity)

Cytotoxicity on adipocyte membranes

The sera from the highly positive turkeys were pooled while the adipocytes were obtained from different fat depots of old layer chicken.

The cytotoxicity assay in this study used the two stages standard protocol of Korzeniewski and Callewaert (1983). The

first stage consisted of the cell processing stage for the release of lactate dehydrogenase (LDH) while the second stage consisted of the determination of LDH itself. This study modified the basic protocol in order to get a certain pattern and was done through 4 consecutive assays (*Table 1*).

Cytotoxicity on red blood cells Blood samples from layer chicken were collected in EDTA tubes and centrifuged at 280 xg for 5 min at 4 °C. The plasma was removed and the compacted red blood cells (RBC) washed 3 times with medium A (medium 199 containing 10 mM Herpes pH 7.4) containing 4 mg/mL glucose. The final pellet was adjusted to 1.6×10^8 cells/mL with the above medium. Incubations were carried out in 1.5 mL eppendorf tubes containing 250 μ L RBC, 250 μ L guinea pig complement (diluted 1:10) and 250 μ L diluted test serum. (The test serum was serially diluted). The mixtures were then incubated for 1 h at 37 °C and overnight at 4 °C. Hemoglobin release was determined by measuring the absorbance of the supernatant in a 96 well microtitre plate on a microplate reader using a wavelength of 600 nm. Total release was determined by incubating a sample of RBC with 0.2% Triton X 100.

Results and discussion

Antigen yield and concentration

The yield and the protein content of the membranes are as shown in *Table 2*.

The yield of adipocyte membranes are significantly different between depots. It is shown here that the subcutaneous plus the abdominal depot yielded less membrane than the subcutaneous fat depot alone. During the digestion it was observed that the abdominal fat was softer and yielded more oil. The protein content however, were almost similar between different depots, an indication that the protein content of membranes are similar irrespective of the depot that it came from.

Table 1. Modifications of the standard procedure for determination of cytotoxicities of antibodies against adipocyte membranes

Steps modified	Assays			
	1	2	3	4
Target cells	Chilled or frozen	Chilled only	Same as 2	Same as 2
Serum (normal and anti) treatment (for destroying endogenous complement)	Heating serum to 56 °C (water bath) and using different levels of the serum	Same as 1	Same as 1	Same as 1
Exogenous complement (assist in cell membrane lysis)	Incubation at room temperature for 30 min before addition	Same as 1	No incubation	Same as 3
Final incubation of target cells, serum, complement and media	30 min at 37 °C (water bath)	Same as 1	2 h at 37 °C (incubator)	Same as 3
Total lysis solution (Triton X-100)	Different levels of Triton X-100 (1, 1.5, 10%)	Triton X-100 = 1%	Same as 2	Same as 2
Reading of LDH release	5 to 30 min after reaction terminated and read at 590 nm	Same as 1	30 min. at 490 nm	Same as 3

Table 2. The yield of adipocytes from different fat deposes and the protein content of their membranes.

Subcutaneous and abdominal fat		Subcutaneous fat	
Adipocytes (%)	Membranes protein concentration (µg/µL)	Adipocytes (%)	Membranes protein concentration (µg/µL)
1) 70.45	} Pooled = 1.30	7) 91.04	} Pooled = 1.16
2) 76.96		8) 88.25	
3) 72.50		9) 87.17	
4) 73.47	} Pooled = 0.80	10) 86.06	} Pooled = 0.95
5) 77.10		11) 86.82	
6) 66.60		12) 86.48	
Av. = ^A 72.85 ± 4.007		Av. = ^B 87.64 ±1.826	

^{A, B}Different superscripts indicate differences at $p < 0.05$

Antibody development

The immunisations of the turkeys with this crude membrane protein induced antibody formation. It is shown that 2 out of 10 turkeys started producing antibodies after the second immunisation and by the third immunisation all the turkeys produced antibodies. Antibody productions become maximum at the third immunisation and dropped slightly until the fifth immunisation

(Figure 1). The maximum antibody production at the fifth immunisation was around dilutions of 1:10⁴ to 1:10⁵ for all immunised turkeys (Figure 2). Therefore, the proteins in the membranes extracted were antigenic to raise very good antibody titre in all the turkeys.

Antibody specificity

Preparation of other membranes The protein content of the organ membranes that were extracted were 5.68, 1.19, 1.76, 1.12, 5.41, 1.58, 1.57, and 4.88 μg for liver, heart, kidney, spleen, brain, muscles, lungs and blood respectively. The values obtained indicated that the organs with the high protein content like the liver, brain and blood may have more membranes surrounding them.

SDS-PAGE and Western Blotting

Separation by SDS-PAGE of plasma membrane proteins from adipose tissues, brain, muscle, liver, kidney, heart, spleen and red blood cells were followed by Western blotting using turkey antibodies against layer chicken adipocyte plasma membranes. Results are as shown in *Plate 1*. Alkaline phosphatase-labelled antibody system was used to detect bound antibodies in order to produce a highly sensitive

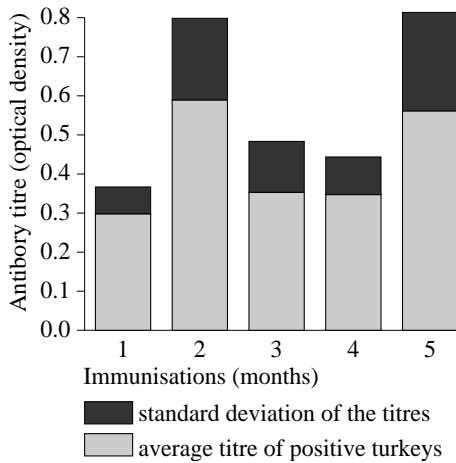


Figure 1. Antibody titres (in optical densities) of positive turkeys after consecutive immunisations

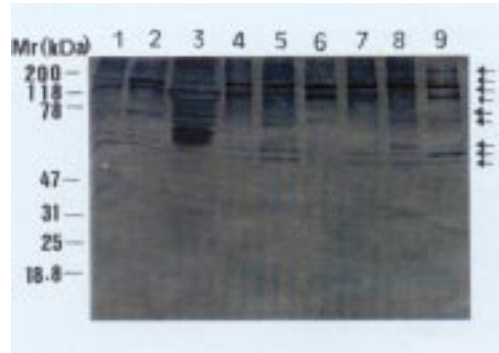


Plate 1. Western blot against proteins prepared from membranes of (1) liver, (2) brain, (3) blood, (4) spleen, (5) kidney, (6) muscles, (7) lungs, (8) heart and (9) adipose tissues (control), using turkey antiserum as primary antibody. The amount of protein loaded per lane was 5 μg

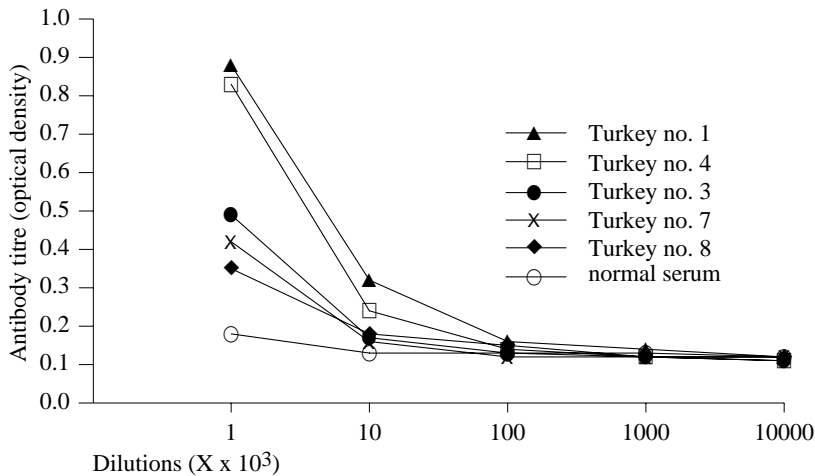


Figure 2. Antibody titre (optical densities) of different dilutions (1:1000) of different turkeys' antisera after the fifth immunisation

response. The results indicated that there are a few reactive proteins in the liver.

However, a number of proteins in the blood, brain, heart, muscles and kidney samples appeared to cross-react with the antibodies.

Comparison of proteins detected in adipocyte membranes (lane 9) resulted in apparently 9 proteins being identified as potential adipocyte antigens. These are 120, 118, 96, 86, 78, 67, 60, 58 and 48 kDa. Two of these proteins, 58 kDa and 96 kDa were reported by Butterwith et al. (1992).

The fact that anti-membrane antibody reacted with proteins from adipocyte membrane indicated that the adipocyte membrane contains proteins that react with these antibodies. However, the reactions of these anti-membrane antibodies to other proteins from the other organs suggest that there may be non-specific binding or these other membranes may contain similar proteins. These interactions need to be investigated further.

Antibody cytotoxicities

Cytotoxicity on adipocyte

membranes The results from assays 1 to 3 did not show any pattern, except for assay 4, which was the last assay to be done (Table 1). The conditions in this assay included the use of antiserum or normal serum, which had been heated to 56 °C in a water bath. Consequently, intact frozen cells plus serum plus media plus complement were incubated at 37 °C in an incubator for 2 h. The LDH was read after 30 minutes on termination of reaction using a 490 nm wavelength.

The results (Figure 3) showed that the cytotoxicities of both the antiserum and normal serum increased with their increasing concentrations. However, the cytotoxic potency of the antiserum was double that of the normal serum. The normal serum only started showing its cytotoxic effect at only 5% of its own concentration and increased slightly with increasing concentrations. The cytotoxic value of antiserum in this study was only about 50% of that seen by

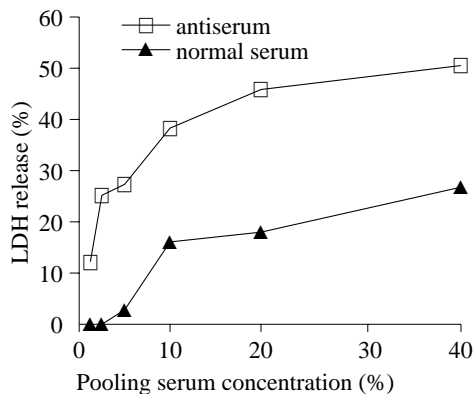


Figure 3. Cytotoxicities (% Lactate Dehydrogenase - LDH released) of different serum concentration of turkey antibodies against chicken adipocytes membranes on chicken adipocytes

Butterwith et al. (1989) who used sheep antibodies against fat cell membranes on chicken adipocytes. Kestin et al. (1993) who also used sheep antibodies against adipocyte membranes found them capable of destroying pig adipocyte in vitro via complement mediated mechanism. The reasons for the lower cytotoxic effect of the turkey against adipocyte membranes here could be many. This may include the modified assay conditions that we used, the different species of animal used (turkey versus sheep) for developing the antibodies, the pooling of antiserum which was done in this experiment, the different antigen preparation and the route of immunisation. It is also a strong possibility that the cytotoxic potency of the antiserum from turkey as seen in this study is less than that of sheep (Butterwith et al. 1989) eventhough both used the same type of antigen. Similarly, Nassar and Hu (1991) who used horse antibodies against adipocyte membranes in sheep noticed less effects in the efficiency of carcass production as compared to Moloney and Allen (1989) who used donkey antibodies against adipocyte membranes in sheep.

Cytotoxicity on red blood cells The contribution of non-specific antibodies to the

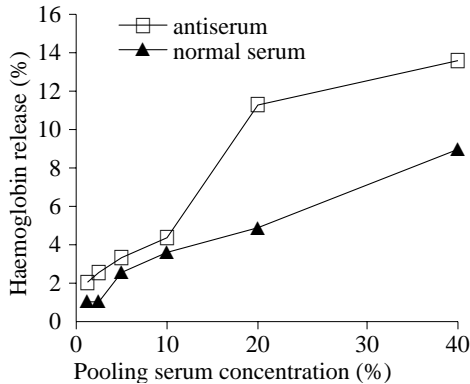


Figure 4. Cytotoxicities (% haemoglobin release) of turkey antibodies against chicken adipocyte membrane on red blood cells (RBC)

cytotoxic response observed on adipocyte membranes was assessed by incubation of the RBC *in vitro*. The results are as shown in Figure 4 above. There was not much difference in percentage haemoglobin release at serum concentration of 10% and below. However, above this concentration the difference in percentage release of haemoglobin between anti-serum and normal serum increased to about 6%. This differs to what had been obtained by Butterwith et al. (1989) where the difference in effects was about 50% between anti-serum and normal serum at 10% serum concentration. It would therefore appear that the anti-serum from sheep as had been used by Butterwith et al. (1989) contained more non-specific antiserum than that obtained from turkeys as what we had used here. The side effects of the chicken turning blue as seen by Butterwith et al. (1989) was also not seen in this experiment, an indication that the turkey antibodies used are less cytotoxic on blood membranes and apparently more specific on the adipocyte membranes. Thus, this may be the reason for the fewer side effects observed here.

Conclusion

It is concluded that the antigens extracted in the form of chicken adipocyte membranes were successful in eliciting high antibody production in turkeys. Thus, this modified

protocol that was used was able to extract adequate membranes to be used as the antigen. However, the yields of membranes would be more if the subcutaneous fat depo instead of the abdominal fat depo were used as the depo for fat tissue extraction.

Consequently, characterisation of the antiserum produced by the turkeys showed that the turkey antibodies reacted mainly with adipocyte proteins (membrane) and were also able to lyse them. These antibodies had very little cytotoxic effects on red blood cell (RBC) membranes. Further investigation is therefore needed for more information with regard to its reaction.

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