Identification and Characterization of Heat-Stable Allergens from *Sarcoptes scabiei*

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ABSTRAK

TARIGAN, S. 2006. Identifikasi dan karakterisasi alergen *Sarcoptes scabiei*. *JITV* 11(1): 52-60.

Hewan atau manusia yang sembuh dari infestasi *Sarcoptes scabiei* memiliki kekebalan atau imun proteksi terhadap reinfestasi tungau. Kekebalan ini diduga berhubungan dengan reaksi hipersensitivitas tipe 1 terhadap alergen yang distimulasi oleh tungau sewaktu infestasi. Oleh karena itu, alergen tersebut diduga mempunyai potensi untuk digunakan sebagai komponen utama sebuah vaksin anti skabies. Penelitian ini bertujuan mengidentifikasi dan mengkarakterisasi alergen sarcoptes tesebut. Untuk itu, sebanyak 645 mg tungau yang diambil dari kambing skabies, dihomogenisasi dalam PBS untuk mendapatkan protein terlarut tungau. Awalnya fraksinasi dilakukan dengan kolom *Q-sepharose* tetapi hasilnya kurang memuaskan sehingga dipilih SDS-PAGE sebagai alternatif. Protein dari gel ditransfer ke dalam membran nitroselulosa, lalu membran dipotong-potong sehingga setiap potongan mengandung protein dengan berat molekul berturut-turut ≥ 90, 80-90, 70-80, 60-70, 50-60, 40-50, 30-40, 25-30, 20-25, 15-20 dan 10-15 kDa. Stabilitas terhadap panas diuji dengan memanaskan suspensi protein pada suhu 60ºC selama 60 menit, sedangkan dialisabilitasnya dianalisis dengan ultramembran yang mempunyai *molecular cut off* 10-kDa. Aktifitas alergen diuji dengan *intradermal tes* menggunakan kambing yang sebelumnya telah dibuat sensitif. Penelitian ini menunjukkan bahwa ekstrak protein tungau merupakan alergen yang sangat kuat karena protein sekecil 1 ng saja sudah mampu menimbulkan reaksi hipersensitivitas yang jelas. Ekstrak tungau mengandung alergen yang tahan panas, sebagian dapat didialisis dan sebagian lagi tidak. Semua fraksi dari kolom Q-*sepharose* mengandung alergen dengan alergenisitas yang hampir sama. Hasil fraksinasi dengan SDS-PAGE menunjukkan bahwa alergen *S. scabiei* mempunyai berat molekul sekitar 35 kDa dan sebagian lagi < 10 kDa. Alergen dengan berat molekul 35 kDa diduga merupakan salah satu anggota alergen kelompok 10 sedangkan yang <10 kDa kemungkinan merupakan alergen hapten.

Kata Kunci: *Sarcoptes scabiei*, Alergen, Tahan Panas, Kelompok 10, Hapten

ABSTRACT

TARIGAN, S. 2006. Identification and characterisation of heat-stable allergens from *Sarcoptes scabiei*. *JITV* 11(1): 52-60.

Animals or human recovered from *Sarcoptes scabiei* infestation acquired protective immunity against reinfestation. The protective immunity is considered to be associated with a type-1-hypersensitivity reaction against allergens instigated by the mites during infestation. It is assumed that these allergens have the potential to be used as the main component of an anti-scabies vaccine. The purpose of this study is to identify and characterise the sarcoptic allergens. For this purpose, 645 mg of mites, collected from mangy goats, were homogenised in PBS to prepare soluble mite proteins. Fractionation of proteins was initially performed on a Q-sepharose column but the results were unsatisfactory. Consequently, SDS PAGE was used as an alternative. Proteins from the gel were transferred onto a nitrocellulose membrane. The membrane was cut into strips so each strip contained proteins with molecular weights of ≥ 90, 80-90, 70-80, 60-70, 50-60, 40-50, 30-40, 25-30, 20-25, 15-20 and 10-15 kDa, respectively. The heat stability of the allergens was determined by heating the suspension at 60ºC for 60 minutes, whereas their dialysability was evaluated using a 10-kDa-cut-off ultramembrane. The activity of the allergens was assayed by an intradermal test on sensitised goats. This study showed that mite protein extract was very potent allergens since mite extract containing as little as 1 ng mite proteins still caused an obvious hypersensitive reaction. The mite extract contained heat-stable, dialysable and non-dialysable allergens. All fractions recovered from a Q-sepharose column contained allergens with almost equal potency. Fractionation with the SDS-PAGE revealed that the allergens had molecular weights of 35 and <10 kDa. The former allergen is assumed to be a member of group 10 allergens, whereas the later belong to haptenic allergens.

Kata Kunci: *Sarcoptes Scabiei*, Allergens, Heat-Stable, Group 10, Hapten

INTRODUCTION

Scabies or sarcoptic mange, which has been known for thousand of years, is currently infesting more than 300 million of people, and causing huge economic losses to the primary industries annually (ARLIAN, 1989; RONCALLI, 1987). The disease might not be a significant problem for people in developed countries but for those living in poor socioeconomic condition in developing world the disease causes a distressful

condition. Clinical and microscopical examination on children living in a displacement camp in Sierra Leone revealed that 67% of the children suffered from the disease (TERRY *et al.*, 2001). About 36% of 34,002 patients visiting a central hospital in Malawi in 1988/1989 contracting the disease. In pigs, the prevalence of the disease is very high even in the developed countries in Europe and North America. A survey conducted in Southern Minnesota revealed that 56% of 50 herds, or 14% of 1500 pigs examined were infested (DAVIES *et al.*, 1996). Similar survey conducted in Northern Spain discovered a prevalence of 86.6% of 67 herds, or 33.7% of 818 pigs examined (GUTIERREZ *et al.*, 1996). Sarcoptic mange is one of the most economically important diseases in goats in Indonesia. The annual reports of provincial livestock services (Dinas Peternakan Propinsi) and the regional disease investigation laboratories (Balai Penyidikan Penyakit Veteriner) usually indicate that sarcoptic mange is the most prevalent disease in goats. This means that the disease appears to be the most prevalent and present all the time wherever goats are raised in Indonesia. The prevalence of the disease in a goat population appears to fluctuate considerably, from <5% to nearly 100%. The mortality rate of the disease is reported to be surprisingly high, 67-100% in young and around 11% in mature goats (BROTOWIJOYO, 1987; MANURUNG *et al.*, 1987).

Despite the fact that the disease is a major global human health problem and causes huge economic losses, no notable advance has been made in the means of controlling the disease. Treatment of diagnosed individuals with acaricides, which is currently the only available means of control, is expensive, unpractical, and only offers a short-term control. Vaccination is considered to be the most attractive means of control but the availability of practical vaccines is still a long way off.

Developing anti-ectoparasite vaccines is a demanding and laborious task. It must start with the identification and purification of the parasite-protein components that will induce protection when immunised into animals. Purification of the protective proteins requires a large amount of parasite as a starting material for biochemical fractionation and a large number of animals for vaccine-challenge trials (WILLADSEN, 1997; WILLADSEN *et al.*, 1989). Obtaining large amount of some parasites especially *S. scabies* is difficult because no *in-vitro* culture system has been available, and collecting mites from infested animals is difficult because the mites are microscopic and live in borrows they make in the skin. In addition to the problem of obtaining sufficient amount of mites, testing the immune protective value of a large number of fractions is even more demanding. Limiting the array of mite proteins only to those having the indication of being protective would be a more feasible approach.

It has been well documented that animals or human recovered from scabies possess protective immunity against reinfestation (ARLIAN *et al.*, 1994; ARLIAN *et al.*, 1995; MELLANBY, 1944; TARIGAN, 2003a). This protective immunity is supposedly associated with immediate hypersensitivity provoked by the mites. This means the mite allergens or mite component provoking the reaction might be protective and therefore might be developed into an effectual vaccine. The sarcoptic allergens, unfortunately, have yet to be identified. This study, therefore, is design to identified and characterised the allergens.

MATERIALS AND METHODS

Extraction of mite proteins

Sarcoptes scabiei mites were cultivated and harvested following the procedures described in our previous studies (TARIGAN, 1998). Briefly, healthy goats were infested with *S. scabiei* and infestation was allowed to progress. Having been severely infected, the animals were euthanised, the superficial skin was scraped, and the mites from the skin scraping were harvested using a specially designed equipment. Approximately 645 mg mites were washed once in 1% SDS solution and twice in PBS, then homogenised in 10 ml PBS, pH 7.4 using a glass homogeniser. The supernatant was collected and its protein concentration was determined by the Bradsford's methods (BRADFORD, 1976) using a commercial kit and bovine serum albumin as a standard (Bio-Rad Laboratories).

Chromatographic fractionation

The soluble mite proteins were desalted against 20 m*M* Tris-HCl, pH 8 (buffer A) using a Sephadex-G25 column (Amersham Biosciences). The desalted proteins were injected into a 5-ml-Q-sepharose column (Amersham Biosciences) attached to an Acta Prime chromatographic system (Amersham Biosciences). Unbound proteins were washed from the column with 10 column volumes of buffer A; whereas bound proteins were eluted consecutively with 0.25 and 1 *M* NaCl in buffer A, each with 5 column volumes. The washed and eluted fractions were collected and their protein concentrations were determined then adjusted to 100 ng/ml. The allergenicity of each fraction was determined after serially diluted in PBS.

Heat-stability and dialysis of sarcoptic allergen

Heat-stability of the allergens was determined by heating the soluble mite proteins in 60ºC water bath for 60 minutes, whereas their dialysability was ascertained by passing the supernatant through a 10-KDa-molecular cut off Amicon centriprep (Amicon Inc.). The heated, dyalisate and filtrate were each diluted serially and its allergenicity was determined.

SDS-PAGE fractionation

Polyacrylamide gel was set up in a Mini-Protean-3 cell and a five-well, 0.75 mm thick comb (Bia-Rad Laboratories). The stacking and separating gels contained 4 and 12% monomer of acrylamide, respectively. The mite proteins were mixed with an equal volume of 2x reduced sample buffer then heated at 95ºC for 5 minutes. The first four wells were filled each with 35 µl of the mixture, whereas, the fifth well was loaded with 15 µl prestained molecular weight markers (Amersham Biosciences). Electrophoresis was performed at 100 V until the leading dye reach about 1.5 cm above the bottom of the gel. After electrophoresis, proteins from the acrylamide gel were transferred onto a nitrocellulose membrane using a transfer buffer consisting of 25 mM Tris, 192 mM glycine, and 20% methanofl in an electrophoretic transfer cell (MiniTransblot, Bio-Rad Laboratories) at 100 volts, 350 mA for 60 minutes.

The membrane was first cut vertically to generate 4 membrane columns containing the mite proteins and 1 column containing the molecular weight standard. The first membrane column containing mite proteins was cut horizontally at 15, 20, 25, 30, 40, 50, 60, 70, 80 and 90 kDa positions. Determination of molecular weight was done according to a procedure described by ROSENBERG (1996). Briefly, the migrating distances of leading dye and each of the molecular weight standard were measured than its relative mobility (Rf), which was the migrating distance of a molecule standard divided by the migrating distance of the leading dye, was calculated. A linier regression equation between log molecular weight and the relative mobility was determined. Based on the regression equation, the position of 15, 20, 25, 30, 40, 50, 60, 70, 80 and 90 kDa on the membrane were marked then the membrane column was cut horizontally at those positions, generating 11 membrane strips containing <15, 15-20, 20-25, 25-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90 and >90 kDa proteins, respectively.

Proteins from each membrane strip were extracted according to the procedure described by ROSENBERG, (1996). Briefly, each membrane strip was incubated in 500 µl of 25% solution of acetonitrile at 37ºC with constant shaking for 3 hours. After the incubation, the tubes were centrifuged at 14, 000 x *g* for 10 minutes and the supernatants were collected. Fresh acetonitrile solution (250 µl) was added to each tube, centrifuged for 5 min and the supernatants were collected and pooled with the previous. The supernatants were lyophilised and the extracted proteins were solubilised in 250 µl PBS. The allergenicity of each extract was determined by an intradermal test.

As the proteins extracted from 30-40 kDa strip were positive in the intradermal test, similar strip from the second membrane column was marked then cut horizontally at 32, 34, 36 and 38 kDa positions under a stereo microscope generating 5 membrane strips containing 30-32, 32-34, 34-36, 36-38, and 38-40 kDa proteins, respectively. Proteins from each strip were eluted followed by intradermal testing as previously.

Intradermal test

Two groups of animals were used in this study. The first group (2 animals) were one year old goats that had never been exposed to scabies previously; whereas the second group consisted of 3 goats that had sarcoptic mange previously but had been completely cured by ivermectin injections.

An intradermal test was performed according to a procedure described previously (MULLER and KIRK, 1976). Briefly, an area on the side chest of goats was carefully clipped to remove all hair and the skin was cleaned and dried. The site of injections were marked and 100 µl of solution to be tested was injected into the superficial dermis. Histamine solution (10 µg/ml) and PBS were used as positive and negative controls, respectively. The reactions was examined and photographed at 10 minutes after the injection.

Biopsy and histological examination

Three sites for intradermal test were prepared on distal half of an auricle of a sensitesed goat. The sites were respectively injected with 100 µl PBS, 100 µl mite extract containing 0.1 µg mite proteins, and 100 µl histamin solution containing 1 µg histamin. Ten minutes after the injection the distal half of the auricle was cut off after location of the incision had previously been locally anaesthetised. The sample was fixed in formalin, prepared for histological slides and stained with haematoxylin and eosin (H&E) following a standard procedure.

RESULTS

Intradermal injection of sensitised animals with the soluble mite proteins produced a cutaneous anaphylactic, immediate or type-1 hypersensitive

reaction, which was characterised macroscopically by rapid formation of localised oedema or wheal, and erythema or flare (Figures 1B, 2). This reaction was comparable to that produced by intradermal injection of histamine solution (Figure 1C). The reaction could not be attributed to the mechanical injury inflicted by the injection since injection with the diluent only (PBS) did not produce any oedematous or erythematous reaction (Figure 1A). Histologically, the reaction was characterised by remarkable thickening of the dermis, severe dilatation of lymphatics and venules, engorgement of blood vessels especially small arteries, separation of collagen fibers and accumulation of oedematous fluids between the separated collagen bundles. In severe reactions, focal haemorrages were also seen (Figures 1E, F). Injection of diluent (PBS) did not produce any histological changes (Figure 1D).

The mite proteins were very powerful allergens since solution containing as little as 1 ng of proteins produced a remarkable hypersensitive reaction when injected intradermally (Figure 2). The proteins was so powerful that unless special precaution was taken during performing the intradermal test, one injection site was easily contaminated by mite proteins overflowing from previous injection sites.

The mite proteins were well separated by the Q-sepharose column into 3 fractions: unbound fraction ($F_{0\%}$), fraction eluted by 0.25*M* NaCl ($F_{25\%}$), and that eluted by 1*M* ($F_{100\%}$). Each fraction contained allergic activity and no significant differences in the allergenicity between fractionated and unfractionated proteins, and within the fractions (Figure 2). The lowest protein concentration at which its allergic activity could still be detected for the unfractionated, $F_{25\%}$ and $F_{100\%}$ was 1 ng/ml, respectively; whereas that for the $F_{0\%}$ was 0.1 ng/ml. This result may indicate that mite protein extract contained multiple allergens.

Treatment of the mite protein solution at 60ºC for 60 minutes did not reduce their allergenicity (Table 1). This means that all or at least the majority of the mite allergens present in the mite extract were heat stable. Ultrafiltration experiment revealed that the sarcoptic allergens consisted dialysable and non-dialysable allergens (Table 1). Since the filter used in this experiment had 10 kDa molecular cut off, the filterable or non-dialyasable allergens therefore should had molecular weights of 10 kDa or less whereas those of dialysate had higher than 10kDa.

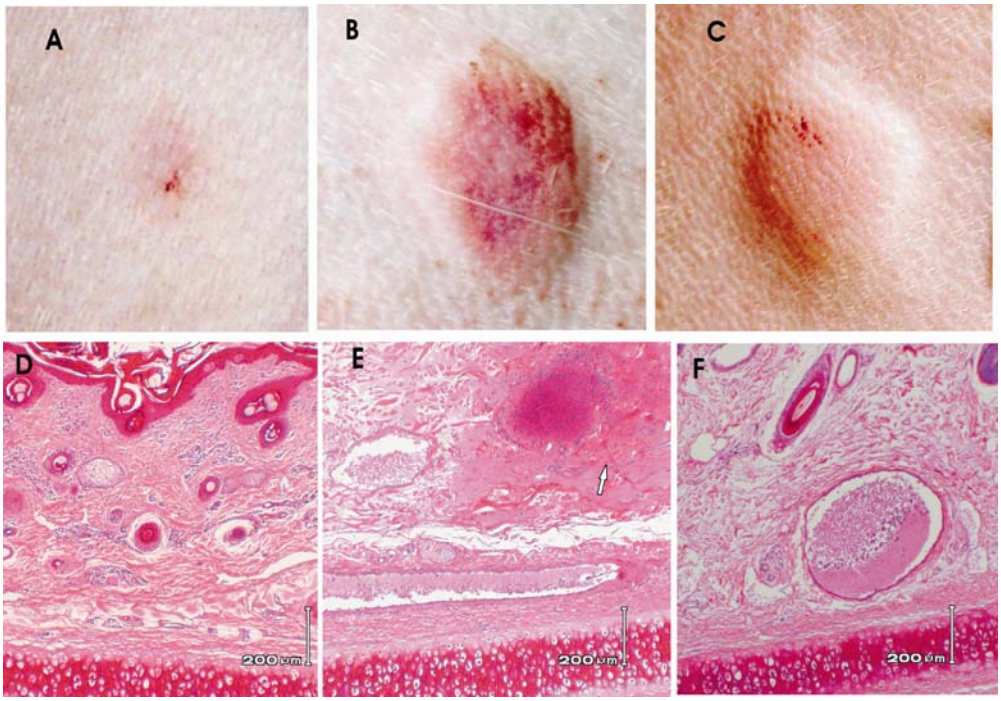

Figure 1 Gross and histology of auricle skin intradermally injected with PBS (A and D), *Sarcoptes scabiei* proteins (B and E), and histamin (C and F). Arrow to indicate haemorrhage

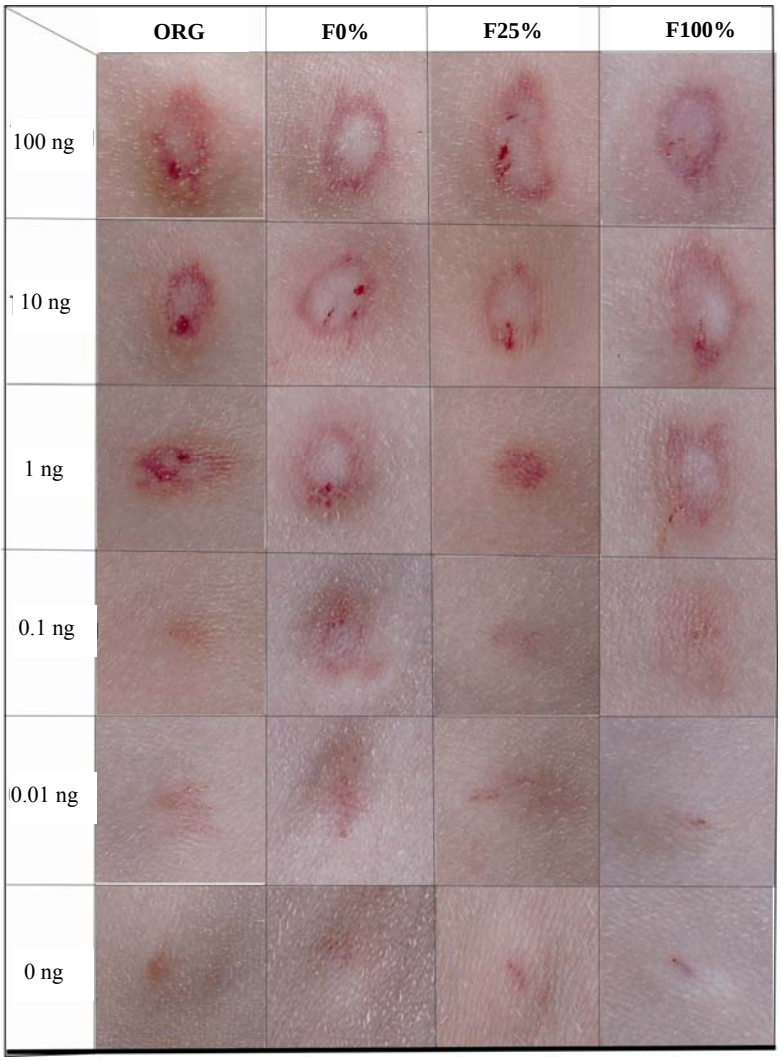

Figure 2. Fractionation of *Sarcoptes scabiei* protein by anion exchange chromatography. *Sarcoptes scabiei* proteins were loaded into Q-sepharose column then eluted with 25% buffer B (1 *M* NaCl in buffer A (Tris-HCl, pH 8) ($F_{25\%}$) and 50% buffer B ($F_{50\%}$). Unfractionated mite proteins and proteins unbound to the column were designated as ORG and $F_{0\%}$, respectively

Table 1. Heat stability and dialyzability of allergenicity of *Sarcoptes scabie* proteins

Dilution	Heated	Unheated	Dialysate	Filtrate
$1:10^{-3}$	++*	++	++	++
$1:10^{-5}$	++	++	++	++
$1:10^{-7}$	++	++	++	++
$1:10^{-9}$	++	++	+-	+-

Note: * = results from two sensitised goats

Having found that the anion exchange chromatography was unsatisfactory means of purifying the sarcoptic allergens and the fact that the allergens were heat stable, SDS PAGE was selected as an alternative. Fractionation on SDS PAGE revealed that the mite extract was composed of proteins with molecular weight from less than 10 kDa to higher than 100 kDa. Those proteins were successfully transferred onto the nitrocellulose membrane and eluted from the membrane after previously had been cut into strips (Figure 3). Allergic activity of protein fraction eluted from each membrane strip was presented in Table 2. As seen from the table, allergic activity was present in fractions $f_{30\text{-}40}$ (proteins with M_r 30-40 kDa) and $f_{<15}$ (proteins with M_r <10 kDa). Further fractionation and assay of the $f_{30\text{-}40}$ revealed that the allergic activity was present in protein fraction with M_r 34-36 kDa.

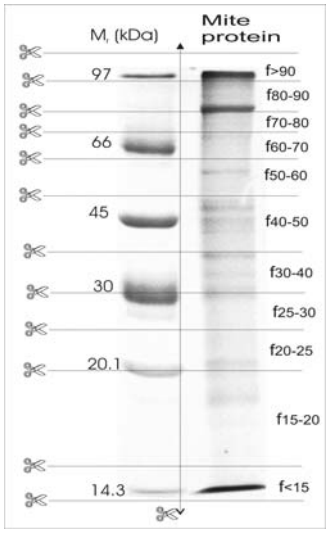

Figure 3. SDS PAGE profile of the soluble mite protein and positions at which the nitrocellulose membrane was cut for protein elution

DISCUSSION

This study shows that intradermal injection of sensitised animals with soluble mite proteins produces cutaneous anaphylaxis or immediate hypersensitivity. The sarcoptic allergens responsible for the reaction are heat stabile consisted of dialysable (≈ 35 kDa) and non-dialysable (<10 kDa) proteins. The reaction is deemed to be the type-1 or immediate hypersensitivity because it developed rapidly (within 10 minutes) and the pathological changes, wheal and flare, were specific for type-1 hypersensitivity. In addition, the mite proteins produced such a reaction only in previously infested or sensitised goats. Intradermal injection of naïve goats with the mite proteins at the same dosages did not produced discernible changes (data not presented). The reaction is also considered to be IgE-dependent since sensitised goats, as demonstrated in our previous studies, have circulating scabies-specific IgE antibodies (TARIGAN, 2004; TARIGAN and HUNTLEY, 2005).

Table 2. Allergic activity of mite protein fractionated with SDS PAGE

Fraction	Allergenicity
>90 kDa	- - -*
80-90 kDa	- - -
70-80 kDa	- - -
60-70 kDa	- - -
50-60 kDa	- - -
40-50 kDa	- - -
30-40 kDa	+ + +
25-30 kDa	- - -
20-25 Kda	- - -
15-20 Kda	- - -
<15 kDa	+ + +

Fraction	Allergenicity
38-40 kDa	- - -*
36-38 kDa	- - -
34-36 kDa	+ + +
32-34 kDa	- - -
30-32 kDa	- - -

Note: * results from 3 goats

The development of cutaneous anaphylactic reaction in human or animals associated with scabies infection has been known for decades. A number of studies describing the reaction in human have been conducted since 1940s when the disease was prevalent in Europe (MELLANBY, 1944). Similar reaction was described several decades later in pigs (SHEAHAN, 1975), dogs (ARLIAN *et al*., 1996), rabbit (ARLIAN *et al*., 1994), and goats (TARIGAN, 2003a; b).

The reactions of infected pigs against intradermal injection of mite extract, as demonstrated by DAVIS and MOON (1990) progress through five phases: (1) sensitisation, (2) delayed hypersensitivity alone, (3) immediate and delayed hypersensitivity together, (4) immediate hypersensitivity alone, and (5) desensitisation. The onset and duration of the delayed and immediate hypersensitivities were reported to be associated with the number of mites infesting the animals. The intensity of a cutaneous hypersensitive reaction against intradermal injection has also been shown to be associated with nutritional status of the animals (SHEAHAN, 1974).

Despite the fact that scabies-associated-cutaneous hypersensitivity has been known for decades, the mite components or allergens responsible for the reaction remain unidentified. Identification and purification of the allergens represent a significant step forward in the development of anti-scabies vaccine because cutaneous hypersensitivity is associated with protection against mite reinfestation (ARLIAN *et al*., 1994; ARLIAN *et al*., 1995; MELLANBY, 1944; TARIGAN, 2003a). Purification of the allergens has been impeded by a number of difficulties. Firstly, biochemical fractionation of the allergens required a large amount of mites which is difficult to obtain because no *in vitro* culture system has so far been available for the mite propagation, and collecting such large amount of mites directly from infected animals is difficult because the mites are very small and live in the burrows they make in the skin. Secondly, the sarcoptic allergens, as shown in this study, are consisted of multiple proteins. Thirdly, intradermal test that used to assay the allergens in the purification process, as used in the study, is time consuming and expensive. In addition, the test is often excessively too sensitive. The presence of the allergen in a fraction to be tested even though its concentration was extremely low would give positive reaction indistinguishable with that produced by fractions having much higher allergen concentration. This is probably the reason why the ion exchange chromatography in this study was unsatisfactory for the purification of the allergens because all fractions after column separation gave positive reaction.

Sarcoptes scabiei has been demonstrated to be antigenically cross reactive with house dust mites *Dermatophagoides pteronyssinus* and *D. farinae* (ARLIAN and MORGAN, 2000; ARLIAN *et al*., 1991; ARLIAN *et al*., 1988; SCHUMANN *et al*., 2001). These astigmatid mites are the major source of house dust allergens triggering allergic diseases in human such as bronchial asthma, perenial rhinitis, and atopic dermatitis. At least 17 proteins (groups 1- 17 allergens) synthesised by the mites have been identified to be associated with allergic disease in human and the molecular characteristics of the allergens have been intensively studied (KAWAMOTO *et al*., 2002; THOMAS and SMITH, 1998). Four allergens, the M-177 apolipoprotein (Group 14), glutathione S-transferase (group 8), paramyosin (Group 11), and serine proteases (group 3) have been identified their homologues in *S. scabiei* cDNA libraries (FISCHER *et al*., 2003; HARUMAL *et al*., 2003). The molecular weights of groups 14, 8, 11 and 3 allergens have been predicted to be 190, 26, 83 and 25 kDa, respectively (KAWAMOTO *et al*., 2002). The approach used in the present study failed to detect allergen with molecular weight similar or close to any of those proteins.

On SDS-PAGE, the mite extract consisted of a great variety of proteins with molecular weight ranged from less than 10 to over 100 kDa. In previous studies it was demonstrated that the mite extract contained more than 10 allergens based on their reactivity with scabies specific IgE antibodies (TARIGAN, 2004; TARIGAN and HUNTLEY, 2005). One probable reason why most of those proteins failed to be identified with the approach used in this study was the fact that the proteins lost their allergenicity due to complete denaturation during SDS PAGE and elution of the proteins from the nitrocellulose membrane. Only those allergens that retained their allergenicity even after complete denaturation were detected in this study. A number of house dust mites, such as groups 1 and 2 allergens, are heat labile which means lost their allergenicity when denatured (KAWAMOTO *et al*., 2002). Similar phenomena are supposed to happen with sarcoptic allergens.

The allergens identified in the present study were unknown which groups they belong to. Based on the similarity of their molecular weights, the one with calculated M_r of 35 kDa might belong to Group 10 allergen which has a M_r of 33 kDa (KAWAMOTO *et al*., 2002). Nevertheless, this group of allergen did not identified in the previous studies (FISCHER *et al*., 2003; HARUMAL *et al*., 2003; MATTSSON *et al*., 2001). Whereas, the non dyalisable allergens with calculated $M_r < 10$ kDa are probably haptenic allergens derived from protease breakdown of high molecular weight allergens. Similar results regarding these low molecule allergens have been documented by previous studies in house dust mites (KAWAMOTO *et al*., 2002).

CONCLUSION

To sum up, animals infested with *S. scabiei* develop a type-1 hypersensitivity. The sarcoptic allergens are multiple proteins consisted of dialyzable and non-dialyzable proteins. The sarcoptic allergens with estimated molecular of about 35 and < 10 kDa have been identified. These allergens which are supposed to be a member of groups10 and haptenic allergens, respectively are heat stable and retain their allergenicity even under stringent denaturation.

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