Discussion

Until now, most of the studies reported on latex allergens were related to proteins in three fractions from ultracentrifuged latex namely rubber particles (RP), C-serum and B-serum. The complete identification of latex allergens from those studies are not yet possible. This also includes unknown background of native allergenic proteins in the latex finished products such as latex glove. Hence, limitation for the study that aims to get rid of allergenic proteins from the products still remains.

Among the three fractions of ultracentrifuged latex, the bottom fraction seems to be the most important as it gives rise to B-serum and the B-serum was previously identified as the major source of immunogenic proteins in latex gloves (Sunderasan and Yeang, 1993; Cardosar *et al.*, 1994). However, when the lutoids bursted out, its B-serum is released and mixed with C-serum. The remaining lutoidic membrane (LM) will, however, remain suspended in the latex system. Our group has found that the LM consists of many proteins associated with lipid bilayer and one is a lectin that can induce RP aggregations (Piyaporn, 2001). It is therefore, the objective of this thesis to study possible involvement of the overlooked bottom fraction membrane (BFM) in latex allergy. In this study, we found that the hydrophobic proteins of BFM can be prepared by extractions with detergent (such as Triton X-100), solvent (such as chloroform-methanol) or ammonia solution. However, the amount and types of extractable proteins were dependent on different method employed. BFM proteins were shown to capable of binding to IgE from subjects with latex allergy and some

shown to have tendency as potent allergens. Similar to RP, when BFM was suspended in ammonia solution, a certain portion of BFM proteins were released into the solution medium and subjected to further degradation along the prolong alkaline treatment. The protein portions that remained with the BFM were found to be more alkaline-stable. Among all proteins from various isolated fractions, the proteins from BFM and RP were more alkaline-stable than those from the C-serum. The C-serum proteins were very susceptible to alkaline-labile and most of them were completely hydrolysed after 15 days of alkaline treatment. In additions, we also found that most of the alkaline-stable proteins in ammoniated latex were originally from BFM. The contamination of BFM proteins into B- and C-sera was found to take place due to freeze-thaw procedure employed.

Under the conventional process used in producing the latex concentrate, final composition of latex concentrate was found to contain not only rubber particles (zone 1) but also certain portion of BFM associated with RP. The associated BFM can, therefore, serve as an additional source of released proteins besides RP. When latex concentrate was make into finished products such as latex glove, the BFM proteins were also detected in the products. Moreover, the BFM proteins were found to be more reactive in binding to anti-glove IgG than those of C-serum and RP.

Part 1. BFM proteins and their allergenicity

The bottom fraction of ultracentrifuged fresh latex consists mainly of lutoid particles with numerous other co-sedimenting particles occupying volumes of 18-36% of latex (Resing, 1955). However, it was found in our study that the BFM protein composition was quite similar to the membrane of purified lutoid particles (Fig. 19). Therefore, the BFM proteins in this study can represent LM proteins or vice versa.

The lutoids are reported to vary in size from 0.5-3 μm and bound by a unit membrane about 80 ºA thick (Dickenson, 1965; Gomez and Moir, 1979). They are flexible, capable of deformation under the influence of complex forming chemicals and can be modified by chemical treatments (Gomez and Southorn, 1969). Due to this property of LM, various procedures used for bursting lutoids should give different structural alternations on the bursted membrane and consequently to the extractable proteins. A hypotonic bursting of lutoids by means of suspending the whole bottom fraction in distilled water and followed by Triton X-100 extraction on the collected membrane was found to be the suitable method in obtaining most numerous LM proteins. Under distilled water hypotonic bursting, the membrane was not disturbed by any foreign chemicals. This is different from an alkaline bursting where the whole bottom fraction was suspended in ammonia solution. It was found that large portions of the 30-35 kDa proteins were partially lost from the membrane and lower amount recovered in the Triton X-100 extract. The latex alkaline bursting condition represents real situation since the ammonia is usually added to freshly collected field latex before latex concentrate processing.

In certain cases, membrane-bound proteins had also been classified as proteolipids such as those found in brain myelin (Folch and Lees, 1951), animal mitochondria (Murakami *et al.*, 1963) and *Hevea* rubber particles (Hasma, 1987). In this study, proteolipids from BFM was extracted by chloroform-methanol in the form of 'fluff'. The proteins associated with fluff could be further extracted by Triton X-100 or SDS. The latter was found to be more effective than the former. The SDS-PAGE of the SDS-extracted BFM proteolipids revealed two major components, 17 kDa and 30-35 kDa proteins. Moreover, if BFM proteolipids or fluff was further treated with alkaline or suspended in alkaline solution before the SDS extraction, more amounts and types of proteins could be obtained (Fig. 27). This indicated there are different types of the integral BFM proteins similar to those described for general membrane by Findley (1990). To remove all of the BFM proteins, various treatments and detergents are required.

As analysed by SDS-PAGE, BFM contained fewer proteins than C-serum and shared certain similarity in pattern to those of B-serum. The similarity in protein pattern between BFM and B-serum is probably due to contaminations by the BFM proteins being released into the B-serum upon freezing and thawing of the bottom fraction. This is evidenced by the fact that similar BFM proteolipids or fluff could also be isolated from the putative aqueous B-serum. Therefore, it should be noted that the B-serum proteins regarding either to this study or the other reports are always contaminated by the released proteins from BFM. However, those very tightly bound hydrophobic BFM proteins may not be

necessary released into the B-serum during freezing and thawing process employed.

In the past, a number of studies have employed immunoblot by using IgE antibodies from latex allergic patients to study their reactivities towards proteins isolated from C-serum, B-serum and rubber particles (Sunderasan *et al.*, 1994; Hasma *et al.*, 1997). In those studies, the proteins from BFM has been overlooked and only the B-serum was used in representing protein component of the bottom fraction of ultracentrifuged fresh latex. But, as earlier described, the BFM contained the variety of proteins and not all equally released into the B-serum. So, the proteins obtained from B-serum could not represent the whole proteins of particles existed in the bottom fraction. By using immunoblot, we have found a number of BFM proteins that showed tendency to be the potent allergens although the results from immunoblot were not always correlated to the results on skin prick tests. The uncorrelated results among immunoblot and skin prick test was also reported by Hasma *et al.* (1997). In this study, the BFM is proposed for the first time as another source of allergenic proteins in addition to those already known RP, C- and B-serum.

Part 2. Alkaline stability studies

The goal of this study is to investigate for better picture on alkaline-stable proteins found in high ammonia (HA) latex concentrate including their respective background origins in fractionated fresh latex. Accordingly, various fractions of fresh latex were isolated and separately treated with ammonia under similar to HA latex concentrate. The alkaline-stable proteins were analysed

under SDS-PAGE and/or immunoblotting and compared to those obtained from alkaline-treated non-rubber or rubber free latex fraction (C-serum and bottom fraction). However, small alkaline-stable polypeptides fragments with effective allergenic/allergenic epitopes may escape from the gel and failed to be detected. To correct for this possible disadvantage, rabbit polyclonal antibodies against total proteins of 60-day-ammoniated latex were raised and used to probe the native proteins of all alkaline-stable proteins in fractionated fresh latex. By using these techniques, alkaline-stable proteins in various fractions of fresh latex were accordingly summarized and discussed.

2.1 Rubber particle (RP) proteins

The RP proteins are those proteins associated with the interfacial layer surrounding the RP. They comprise mainly of two tightly bound anionic proteins with molecular weight of 14 and 24 kDa and with pI ranging between 3.5 to 6.0 (Hasma, 1994). The 14 kDa protein (Hev b 1) and 24 kDa (Hev b 3) have been recognized as latex allergens with specific binding capacity towards serum IgE antibodies from patients with spina bifida (Czuppon *et al.*, 1993; Yeang *et al.*, 1996; Alenius *et al.*, 1996b).

The RP proteins are quite hydrophobic in nature found to be partially released under alkaline condition. Most of the released protein portion could retain their stability in the ammonia solution for only up to 4 months. This is unlike the stable RP-bound forms. This result was in agreement with earlier reports by Hasma (1992) and Hasma and Amir-Hashim (1997) on the detection of RP proteins in both serum and rubber fractions of preserved HA latex concentrate. However, the 24 kDa protein was found to be more alkaline-labile

than the 14 kDa one by exhibiting faster degradation rate upon further incubation under alkaline condition. This is in agreement with earlier observation by Yeang *et al.* (1996) on fragmentation of the small rubber particle protein, 24 kDa, upon storage at -20ºC or by reacting with the B-serum.

2.2 C-serum proteins

The aqueous C-serum contains a variety of different proteins, both anionic and cationic proteins with pI ranging from 3.5 to 9.5 (Hasma, 1994). Some of the C-serum proteins were shown to be latex allergens. These include acidic protein, Hev b 5 (Akasawa *et al.*, 1996a; Slater *et al.*, 1996), patatin like protein, Hev b 7 (Beezhold *et al.*, 1994) and manganese superoxide dismutase, Hev b 10 (Posch *et al.*, 1997) with molecular weight of 16, 46, 51 and 26 kDa, respectively. These C-serum proteins were found to be recognized by IgE antibodies in sera of patients with latex allergy.

In contrast to RP proteins, the C-serum proteins were alkaline-labile and most of them were undetectable under SDS-PAGE after 15 days of alkaline treatment (Fig. 22). There was apparently one C-serum protein of about 34 kDa that remained stable under alkaline condition as shown on SDS-PAGE. This is however, doubtful whether it is native to the C-serum proteins or being released from other sources such as B-serum or BFM. Contamination of B-serum in C-serum due to ruptured lutoids in the process of collection and centrifugation of fresh latex had been reported by Cardosar *et al.* (1994). In our study, by using rabbit antiserum against BFM proteins (30-35 kDa), the contaminated BFM proteins were also found in C-serum (Fig. 33B). Therefore, the chance was very

limited for the native C-serum proteins to survive under alkaline condition and become allergen candidates in latex finished products.

2.3 B-serum proteins

The B-serum prepared from bottom fraction organelles normally contains dissolved organic substances and suspended fibril of a protein nature. Similar to C-serum, the B-serum consists of a variety of anionic and cationic proteins with pI ranging from 3.5-9.5 (Hasma, 1994). Several B-serum allergenic proteins with strong IgE binding property had been reported. These different allergenic proteins included basic β-1,3-glucanase of 36 kDa or Hev b 2 (Breton *et al.*, 1995; Sunderasan *et al.*, 1995) and microhelix component with a molecular size of approximately 50-57 kDa or Hev b 4 (Sunderasan *et al.*, 1995). In addition, prohevein of 20kDa (Hev b 6.01) and its post–translationally processed N-terminal fragment of 4.7 kDa or hevein (Hev b 6.02) and the C-terminal domain of 14 kDa (Hev b 6.03) were documented as latex allergens (Alenius *et al.*, 1996a; Beezhold *et al.*, 1997; Chen *et al.*, 1997b). Only the N-terminal fragment of prohevein (hevein) and prohevein itself were reported as major allergens in adult health care workers (Chen *et al.*, 1997; Alenius *et al.*, 1995a).

As earlier described, the BFM can be released into the B-serum during freezing and thawing of the lutoid particles. Consequently, the alkaline-stable proteins found in B-serum were doubtful whether they belong to native B-serum proteins or those proteins released from the BFM or both the mixture of them. Moreover, the immunoblot results, using rabbit antiserum against BFM and human serum from subject showing positive SPT towards BFM proteins (Fig. 31

and 32), revealed that the remaining alkaline-stable soluble proteins found after alkaline treatment of a mixture containing non-rubber fractions (C-serum and bottom fraction organelles) were likely to be derived from the BFM rather than the native B-serum proteins.

2.4 Bottom fraction membrane (BFM) proteins

The BFM proteins represent those proteins associated with lipid bilayer of single and double membrane-bound organelles of lutoids and Frey-Wyssling, respectively. They are hydrophobic and extractable by ammonia water, detergent or organic solvent. The fresh BFM consists of cationic and anionic proteins with pI ranging from 4.3 to 9.5 which similar to B-serum proteins (Hasma, 1994). After alkaline treatment for 60 days, only acidic proteins with pI ranging from 4.7 to 6.7 were observed. The alkaline stability of acidic proteins found in HA latex concentrate and glove extracts was also reported by Hasma and Amir-Hashim (1997).

For allergenicity, the alkaline-stable proteins, both released into the solution media and retained with the sedimented BFM, were recognized by IgE from patient with latex allergy. This result thus suggested that these BFM proteins were likely to persist and become allergen candidates in latex finished products.

Addition to the alkaline-stable property of BFM proteins, they are also tolerated to the heat used in the process of prevulcanization. Associations of BFM within membrane lipid bilayers or with lipids in the form of micelles are expected to be the essential factor contribute to their alkaline stability and heat tolerance.

Part 3. Latex concentrate

Due to changes in latex compositions during the preparation of latex concentrate, the protein composition of latex concentrate, therefore, should be depended on the remaining composition of latex concentrate instead of those composition of fresh latex. In this study, we found that RP (zone 1) was the remaining major component of latex concentrate whereas the other components such as Frey-Wyssling particles were removed into sludge and skim latex. However, it was expected that the RP in latex concentrate should also associate with certain portion of LM. The association of LM with RP is possible to take place during the rupture of lutoids by mechanism involved in coagulum formation as studied and reported by Piyaporn (2002). Therefore, when the latex was further concentrated by mean of centrifugation, the LM should remain associated with RP and become major component of latex concentrate. The association of RP and LM was shown in the analysis of proteins on different storage time of HA latex concentrate. By using rabbit antiserum against BFM proteins, BFM proteins were detected in both of rubber and serum fraction of latex concentrate (Fig. 42). The presence of BFM and RP proteins as the major component of latex concentrate also in agreement with the results on alkaline stability studies where BFM and RP were found as major sources of alkaline-stable proteins (Fig. 34).

Part 4. Latex glove

Latex glove products are produced from chemical-compounded latex concentrate. The Western-immunoblot of extractable proteins obtained from

different brands of natural latex gloves, using rabbit antiserum against BFM proteins, revealed the presence of proteins in latex gloves. The amount found was more or less dependent on the manufacture process. These results thus indicated that when latex concentrate was converted into glove, glove protein compositions should resemble those found in original latex concentrate. However, extractable proteins from extensively washed latex glove should be represent mainly released proteins from RP or its associated BFM/LM.

The indirect ELISA and immunoblot results, using rabbit antiserum directed against proteins eluted from ultra-low protein gloves, showed very high antibody recognition on BFM and B-serum, minimal on the C-serum and non on the RP. This confirmed that a large amount of glove proteins were derived from BFM. The less immunogenic responses of C-serum and RP to anti-glove IgG was had earlier been reported by Sunderasan and Yeang (1993) and Cardosa *et al.* (1994). Moreover, the results also suggested that the alkaline-stable proteins of RP were easier to be removed under extensive washing than those of the BFM which retained in ultra-low protein gloves.