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Correlation between Total Extractable Proteins and Allergen Levels of Natural Rubber Latex Gloves

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Certain proteins or peptides eluting from natural rubber (NR) latex products can cause immediate hypersensitivity reactions (Type I allergy) in subjects sensitised to them The amount of total extractable proteins in manufactured latex products is believed to reflect reasonably well their corresponding allergenic protein level, but only a few studies have been published to substantiate this. The aim of the present study is to compare a widely used total protein measurement assay, namely, the RRIM modified Lowry test (EP_{RRIM}), to latex allergen analysis, carried out by specific IgE-ELISA-inhibition tests. A series of 46 widely marketed medical NR latex gloves was investigated. Their EP_{RRIM} values ranged from < 20 µg/g to 1290 µg/g, and their allergen content varied from < 1 AU/ml to 570 AU/ml In the measurement of allergen contents, the reference allergen mixture was prepared from serum proteins of fresh Hevea *latex, and IgE antibodies were sourced from both adults and* spina bifida *children sensitive to latex Results showed that the allergen levels were very well correlated with the total extractable protein contents (coefficient r = 0.89, P<0 001, n = 46) With the exception of a few, gloves with high total extractable proteins were generally found to have high allergen contents, and* vice versa. *Gloves with EP_{RRIM} levels of 0.1 mg/g or 100 µg/g and below always had very low allergen contents (< 9 AU/ml).*

These findings are consistent with those shown by the in-vivo *skin-prick test reported earlier. More importantly, they confirm the very low allergen levels observed at EP_{RRIM} levels of about 100 µg/g and lower Such information provides useful guideline for the manufacturing of reduced risk NR latex gloves.*

Type I allergy affecting certain individuals through the use of some NR latex products, has caused great concern among the latex product manufacturers. Emphasis has since been placed on the production of latex devices with better biocompatibility. Many attempts have therefore been made to reduce their total extractable proteins shown to be implicated in

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the allergy reaction[1,2]. A number of effective methods for such reduction have subsequently been developed in Malaysia. These include the use of low protein latices[3-5], suitable leaching protocols during processing[4,6], enzyme treatment[7], chlorination of the finished products[8] and polymer coating. When applied under suitable conditions, the extractable protein fraction can be effectively reduced to a very low level.

It is noteworthy that the residual extractable fraction of latex products may consist of both the allergenic proteins (*i.e.* capable of binding to IgE antibodies) and non-allergenic proteins, the proportion of which may vary from product to product. It is, therefore, of great importance that reduced levels of extractable proteins reflect the reduced allergen level of the final products. The present work was thus undertaken to study the correlation between total extractable proteins and allergen contents of latex gloves. Results were also compared with those reported earlier[9] on the relationship between allergic response elicited in latex hypersensitive subjects and total extractable proteins of latex gloves.

METHODS

Extractable Protein Content — RRIM Modified Lowry Method [*Malaysian Standard Test Method MS 1392 : 1996 (P)*][10]

Protein extraction. Cut pieces, of 7 cm x 7 cm each from palm area of glove sample were extracted in 0.01 *M* phosphate buffered saline at pH 7.4 (1 g/5 ml) at 23°C for 3 h with agitations at 30 min intervals. The clear extract was obtained after removing any insoluble matter that might be present by centrifugation.

Protein precipitation. Proteins in the extracts were precipitated using trichloroacetic acid (4.4%, w/v) and phosphotungstic acid (0.2%, w/v). The precipitated proteins were then sedimented by centrifugation at 10 000 x g for 30 min. The resulting protein pellet was redissolved in minimum volume of 0.2 *M* sodium hydroxide (1 ml - 6 ml) after removal of the supernatant containing interfering substances.

Modified Lowry microassay. 0.8 ml aliquot of each redissolved protein solution was treated with 0.3 ml of a reagent containing 6% sodium carbonate and 1.5% copper sulphate in 3% sodium citrate (carbonate: sulphate = 10 : 0.2). After allowing the mixture to stand for 10 minutes, 0.1 ml of 72% 2 *N* Folin reagent was added. Colour was allowed to develop at room temperature for 30 min, and its absorbance at 750 nm was recorded. If precipitation occurred at this stage, further centrifugation was carried out to give clear solution for the colorimetric measurements. Results were read against a standard Bovine Serum Albumin (BSA) calibration curve and converted to µg/g or mg/g of gloves, taking into consideration the weight of sample extracted and the volume used in each case.

Allergen Content—ELISA-inhibition Test[11]

Latex serum proteins containing the allergens. Frozen (–70°C) non-ammoniated *Hevea brasiliensis* latex harvested freshly from the trees under chilled conditions, was thawed and centrifuged to give a clear serum containing latex allergens (NRL serum protein concentration 10 mg/ml, as measured by Lowry assay). It was diluted to a protein concentration of 20 µg/ml in 50 mM carbonate buffer at pH 9.6 and applied onto polystyrene microtitre plate (100 µl per well, Nunc, Denmark),

incubated at room temperature for 3 h. The wells were emptied and post-coated with 1% human serum albumin in 50 mM carbonate buffer.

Inhibition and immunoassay. The IgE serum pool for the inhibition reaction consisted of carefully characterised sera from both latex allergic adults (n=3) and latex allergic children with *spina bifida* (n=3). Optimally diluted IgE serum pool was mixed with equal volume of each of the serially diluted glove extract (both in phosphate buffered saline-Tween-human serum albumin) and the mixture incubated for 1 h at room temperature. The inhibited mixtures were then introduced into the coated microtitre plate and incubated for a further 2 h at room temperature. After appropriate washes, the bound IgE was detected by biotinylated goat anti-human IgE (Vector) and streptavidin-conjugated alkaline phosphatase (Bio-Rad). Intensity of colour formed upon reaction with the substrate development solution (Sigma) was read at 405 nm.

A standard curve for the inhibition reaction was obtained, based on serial 10-fold dilutions of the NRL serum containing 10 mg/ml of proteins to which 100 000 arbitrary allergen units (AU/ml) were assigned. OD reading of each test sample was converted to these units from the standard curve.

RESULTS

In the present study, relationship between total extractable proteins (EP_{RRIM}), as determined by the RRIM modified Lowry test, and allergen content/activity, as assessed by the ELISA-inhibition test, was investigated. The reference allergen mixture consisted of serum proteins from fresh *Hevea* latex, while IgE antibodies were sourced from both adults and *spina bifida* children who showed sensitivity to latex. Allergen measurements so generated using these reference mixtures have been shown to be highly correlated to the allergic response by the skin prick test (correlation coefficient $r = 0.94$, $P < 0.001$, $n = 20$)[12], indicating the reliability of the test for allergen quantitation. Accordingly, allergen content of < 10 AU/ml is low, 10 –100 AU/ml is moderate, and > 100 AU/ml is high.

A total of 46 commercially available brands of medical latex gloves of which 11 were powder-free, were examined. Their extractable protein (EP_{RRIM}) content varied from as low as less than 20 μg/g (or < 0.02 mg/g) to as high as 1290 μg/g (or 1.290 mg/g), and their allergen content ranged from < 1 AU/ml to 570 AU/ml (*Table 1*). Median values of the gloves samples were 485 μg/g and 117 AU/ml, respectively. Generally, gloves with high protein values had high allergen contents and *vice versa*. However, there were some exceptions, such as in the case of samples No.16, 20 and 22 showing higher allergen contents than the trend depicted. Similarly, samples No.11, 21 and 26 indicated lower allergen levels than expected. These could well be attributed to variability in protein composition of the residual fraction in the samples concerned, due to marked differences in the processing conditions employed. It is most apparent that at EP_{RRIM} levels of about 100 μg/g or 0.1 mg/g and less, the corresponding allergen contents are consistently and remarkably low at < 9 AU/ml (*Figure 1*). This latter group of gloves consisted of all the 11 powder free gloves examined, in addition to 3 powdered ones. Statistical analysis revealed that the two parameters are well correlated, with the coefficient of correlation $r = 0.89$, $P < 0.001$ (*Figure 2*).

TABLE 1. TOTAL EXTRACTABLE PROTEIN AND ALLERGEN LEVELS IN 46 BRANDS OF NR LATEX MEDICAL GLOVES

Glove brand (Sample No.)	Total extractable protein content		Allergen level
	µg/ml of extract	µg/g of glove	AU/ml extract
1	258	1290	510
2	243	1215	570
3	229	1145	410
4	228	1140	323
5	221	1105	378
6	219	1095	299
7	211	1055	350
8	201	1005	378
9	197	985	239
10	184	920	233
11	183	915	125
12	178	890	307
13	175	875	212
14	167	835	350
15	148	740	264
16	144	720	510
17	142	710	257
18	130	650	188
19	129	645	239
20	121	605	388
21	109	545	105
22	106	530	496
23	102	510	212
24	91	457	145
25	89	445	212
26	86	430	81
27	77	385	197
28	69	345	135
29	65	325	128
30	51	255	128

TABLE 1. TOTAL EXTRACTABLE PROTEIN AND ALLERGEN LEVELS IN 46 BRANDS OF NR LATEX MEDICAL GLOVES (CONT.)

Glove brand (Sample No.)	Total extractable protein content µg/ml of extract	µg/g of glove	Allergen level AU/ml extract
31	48	240	115
32*	21	105	4
33	16	80	3
34	16	80	8
35	13	65	5
36	12	60	2
37*	12	60	8
38*	9	45	5
39*	9	45	4
40*	8	40	4
41*	8	40	5
42*	6	30	3
43*	5	25	2
44*	4	20	< 1
45*	< 4	< 20	3
46*	< 4	< 20	3

*Powder free gloves

Extraction ratio: 5 ml of water per gram of gloves

Allergen levels[12]: < 10 AU/ml – Low; 10 to 100 AU/ml – Moderate; > 100 AU/ml – High

DISCUSSION

Allergenicity

Allergenicity or allergic potential of latex products with reference to latex protein allergy has recently become a parameter of importance in the manufacturing of safer latex articles of low allergen quality, particularly the medical devices. There are, however, no standardised methods for such measurement to-date. Several tests, most of them competitive immunoassays, are commonly employed by various laboratories in the West. These include mainly the radioallergosorbent test with inhibition (RAST-inhibition)[13,14] and the enzyme-linked immunosorbent test with inhibition (ELISA-inhibition)[12]. In these *in-vitro* tests, latex allergens are quantified by allowing the soluble latex allergens in the sample extract to compete with a reference allergen mixture adsorbed on a solid phase, for the binding sites of a pool of latex-specific human antibodies. The amount of the IgE latex-specific antibodies bound to

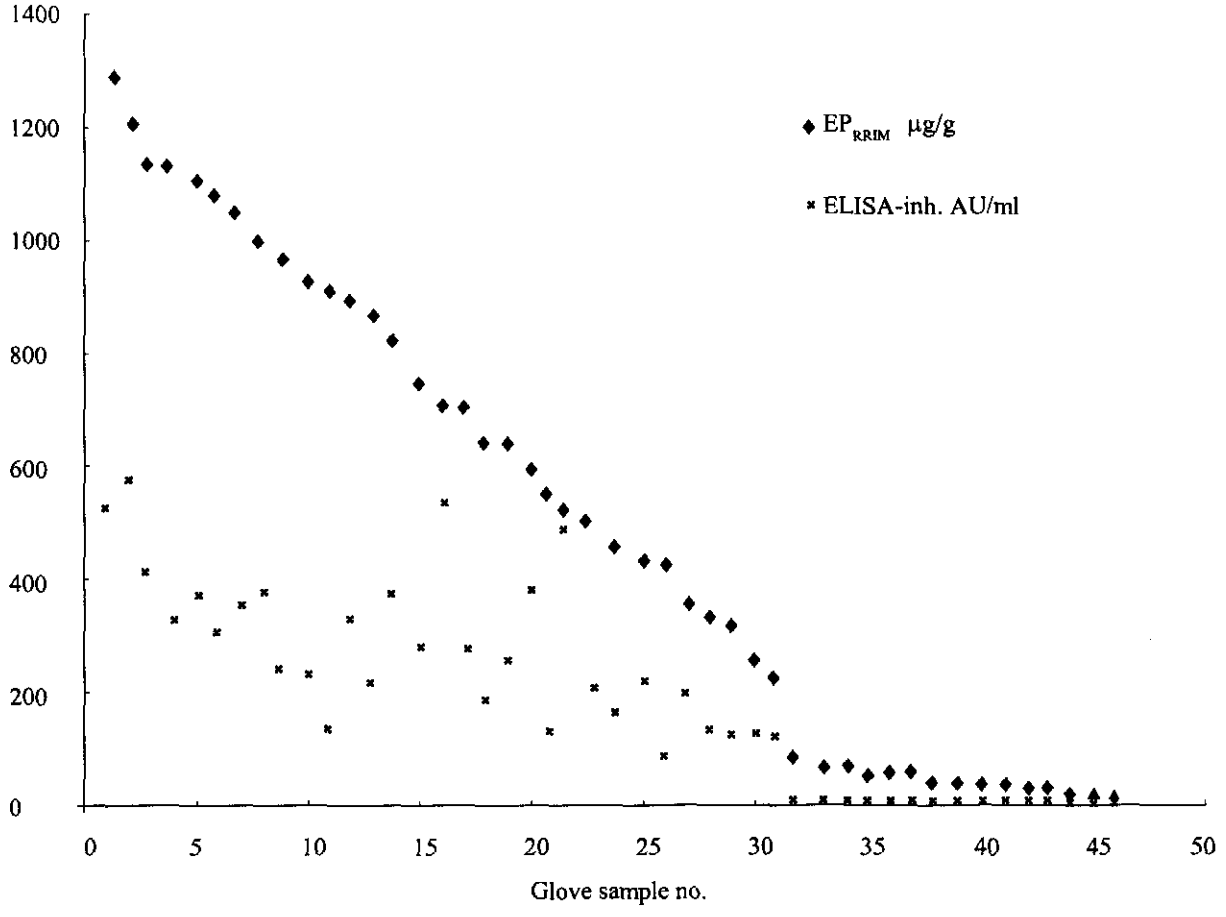

Figure 1. Total extractable proteins (EP_{RRIM}), as determined by RRIM modified Lowry test, and the corresponding allergen content, as assessed by the ELISA-inhibition test, for 46 brands of medical latex gloves.

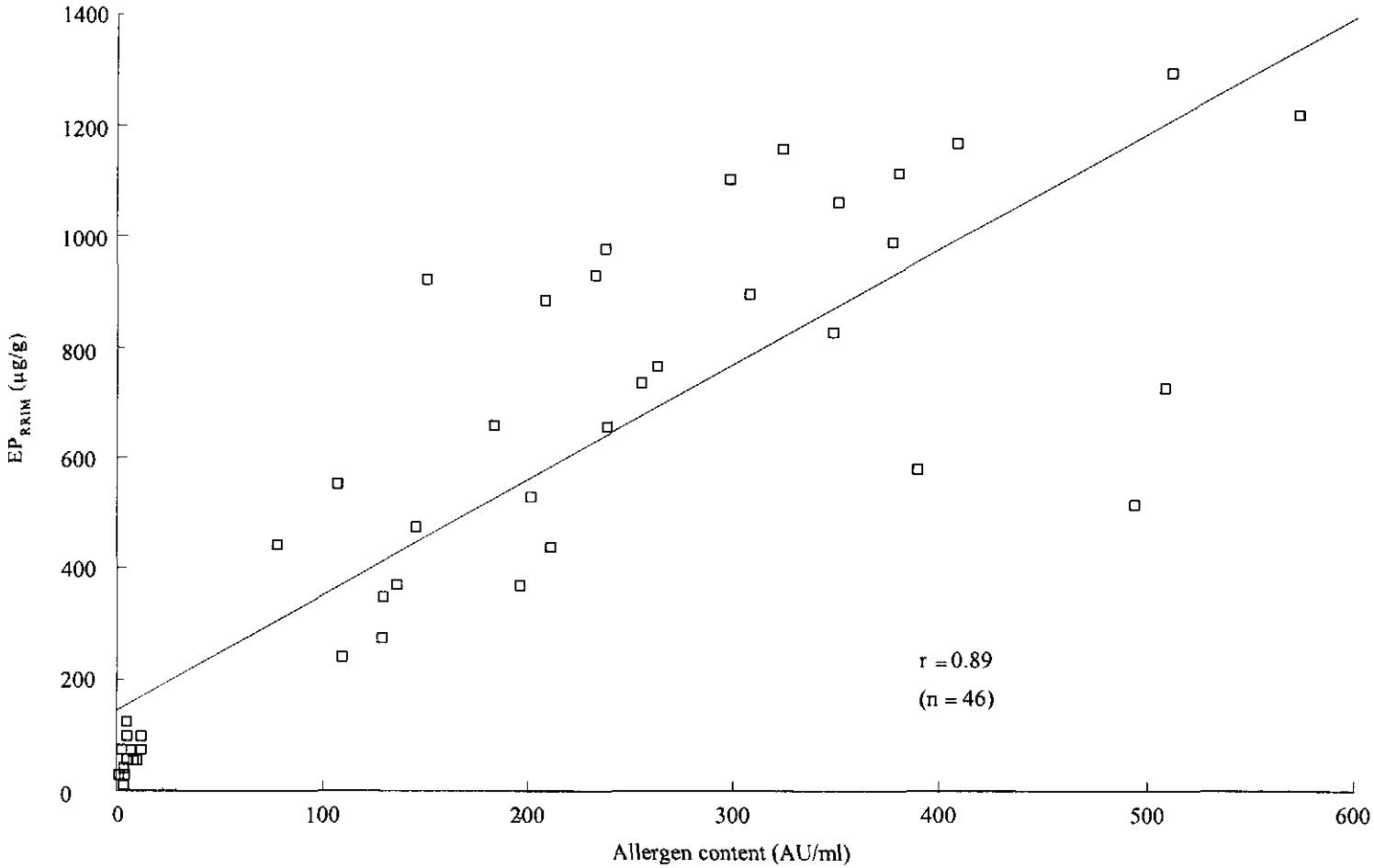

Figure 2. Correlation between total extractable protein contents as determined by the RRIM modified Lowry test, and their corresponding allergen levels as assessed by the IgE ELISA-inhibition assay, for 46 commercially available medical NR latex gloves.

the solid phase is determined, and is inversely proportional to the quantity of latex allergens in the test sample. Resulting measurements are expressed as allergen content/activity in AU/ml. For effective measurements, the reference latex protein standard should contain all relevant allergens, and the reference serum pool should comprise a complement of the corresponding latex specific IgE antibodies. Unfortunately, such standard allergen mixture and IgE serum pool have yet to be developed. In view of this, absolute values of these measurements may vary when either or both the reference allergen mixture and the IgE antibodies used differ. Hence, there is a need to correlate results obtained between laboratories when latex allergens and IgE antibodies from different sources are used.

On the other hand, allergenicity can be specifically evaluated by the *in-vivo* clinical skin prick test, by assessing the allergic response elicited by the protein extract in latex hypersensitive subjects[9,12,14,15]. This test is both sensitive and specific. But the availability of latex sensitive persons is essential. This requirement is not always easily met, especially in latex product manufacturing countries such as Malaysia where prevalence even among the high risk groups has been shown to be very low[16].

Total Extractable Proteins

By far, the colorimetric measurement of total extractable proteins in latex devices offers a relatively simple procedure, involving standard chemicals which can be obtained easily. It has therefore, been adopted for routine monitoring of protein reduction during manufacturing. The modified Lowry microassay protocol which is often used consists of three parts, namely, protein extraction, protein precipitation and the colorimetric microassay. Only the commercially available chemicals are used in this test, and the testing time is (excluding protein extraction time) 2 h – 3 h, as compared to 1–2 days for the immunoassays. Results are expressed in mg/g or μg/g of test sample, with reference to a standard protein. One drawback of this test concerns the fact that it measures all the extractable proteins some of which may not be allergenic. Therefore, for the values generated to be meaningful, they should be related to the allergenicity or the allergen levels.

Relationship

There had been some speculations that total extractable proteins were not correlated to their allergenicity or allergen contents. However, this has been shown to be not so. A good correlation between the two parameters has in fact been shown by Yip *et al.*[9], who assessed the skin prick test allergic response elicited by 39 glove extracts with EP_{RRIM} varying from < 20 μg/g to >1000 μg/g, in a total of 59 latex hypersensitive subjects. The coefficient of correlation, 'r' was 0.83 at $P < 0.001$. This is consistent with the report by Yunginger *et al.*[13] who indicated a significant correlation between extractable protein content of 71 latex gloves, as determined by a modified ninhydrin method, and their allergen levels as assessed by IgE-RAST inhibition immunoassay. Further substantiation is now obtained in the present study which demonstrated a highly significant correlation between the total extractable proteins and the allergen levels of latex gloves, as evaluated by the IgE-ELISA-inhibition immunoassay.

It has been shown that soluble proteins migrate towards the surface of a latex film[17,18]

during the manufacturing process of latex-dipped products. The degree of removal of these proteins from the surface is very much influenced by the processing conditions employed, some being more effective than others[19]. In view of the fact that the residual extractable proteins may not all be allergenic, the doubt often arises as to whether there is any preference in the removal between the non-allergenic and allergenic proteins or among the various allergenic proteins from this fraction. The relationships observed between total extractable proteins and allergen levels and allergenicity[9] suggest that reduction affects both types of proteins, and the existence of some preferences has also been implied.

It is also of interest to note that when reduction reaches a very low EP level of about 100 μg/g and less, evidence strongly indicated that the amount of allergens present, if any, is often too little to facilitate substantial binding with latex-specific IgE or elicit any allergic response in latex hypersensitive persons, as demonstrated by the tests conducted. This is regardless of whether the gloves are powdered or powder free. Hence latex products with such low extractable protein levels can be considered to be of low risk to users. The availability and use of such latex products are expected to reduce or even to prevent further sensitisation. However, it should be stressed that there is a small number of highly atopic people who are sensitive to a great number of allergens. For these subjects, even minute amounts of allergens can elicit hypersensitivity reactions, implying avoidance of all the relevant allergens should be recommended.

It may be mentioned that since absolute values of total extractable proteins generated by different colorimetric methods are not fully comparable[20], results of the present study are relevant only to those determined by the RRIM modified Lowry test. This has consequently rendered the test a convenient and a useful one for monitoring purposes in the manufacture of low protein latex products.

CONCLUSION

Total extractable protein contents of latex gloves (EP_{RRIM}), as determined by the RRIM modified Lowry test, have been found to correlate well with their allergen contents as assessed by the IgE-ELISA-inhibition test. Gloves with high total extractable protein contents are generally associated with high allergen contents, while those with low total extractable proteins tend to have low allergen contents. Protein levels with minimal allergen content/activity have been identified to be about 100 μg/g and lower. These findings are highly consistent with those reported for EP_{RRIM} values and allergic responses by the skin prick test. Such information provides very useful guidelines not only for the manufacture of the more bio-friendly low protein latex products, but also for users in their selection of gloves.

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