Natural rubber latex (NRL) waste protein purified at various pH condition and metal extraction studies

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Abstract. Purification of NRL protein was accompanied by extraction of waste proteins using salting out and multiple centrifugation methods. Both methods contributes to varying degree of yield and purified protein characteristics. However both methods produces protein that can bind metal efficiently. Conventionally, salting out method was used and it is uncommon to use multiple centrifuge to extract protein. The key aspects discussed here, were on how the pH condition exposed to the NRL waste leads to variation in hevea protein extracted amount and how metal binding featured in FTIR spectroscopy. The purified protein were reacted with metal solution of different strength to study the binding characteristics. Molecular weight cut-off (MWCO) size of dialyzing tube shows greater effect on the final protein extracted amount by about 50% when smaller size were used. Acidic condition favors part of proteins from waste in purification while some favors basic condition. Standard salting out method shows consistent profile in extracting metal compared to multiple centrifugation method from 30 to 80%.

1. Introduction
NRL waste collected from coagulated skim rubber serum was used as a starting material. The rejected liquid from centrifugation still containing 4-8 % DRC, called skim latex. Wastewater from skimming process is highly acidic with pH 2.0-4.5, high in COD and sulfate, improper management of this wastewater has long caused water and air pollution to nearby communities [1, 2]. This plant wastewater contains hev proteins which is well known for the allergen characteristics. The physico-chemical characterization of skim serum effluent revealed that the effluent contains many hazardous constituents and also contains significant amount of non-rubber which includes proteins, sugar, carotenoids and very little amount of uncoagulated latex. These constituents are excellent substrates for the proliferation of microorganism generating objectionable odor [3]. Those hev proteins were purified via standard salting out method.

These proteins can be extracted by various methods. Ammonium sulphate purification or also called salting out is the common method used beside acetone and other alcohol base solvent methods. An antimicrobial protein, hevein was extracted from the bottom fraction after centrifugation and purified by acetone fractionation [4]. Alcohol solvent reduces the dielectric constant and subsequently reduces solubility by lowering protein-solvent interactions. The drawback of using this common
method is the removal of chemical or salt used. This study focuses on the comparison of standard ammonium sulphate precipitation with multiple centrifugation method on freeze dried weight and the comparison of extracted proteins from both said methods on metal extraction capabilities. Waste from NRL concentrate processing plant was extracted using standard ammonium sulphate precipitation method where saturated liquid ammonium sulphate was used. Another method only used multiple centrifugation to extract the proteins metal ions and proven to be effective in the removal of Pb\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\), Cr\(^{6+}\), and Zn\(^{2+}\) [5]. Previous researched had employed electro-chemical treatment of skim serum effluent from natural rubber latex centrifuging units and which can be used as a basis for metal-wastewater interaction. Electrochemical treatment is a non-biological and chemical free process, the main reagent is the electron which is ‘clean reagent’[3]. Other than that a method used to treat metal is biosorption which is relatively new process for the removal of contaminants. Biosorbent from agricultural waste have been proved for the effective removal of hexavalent chromium from aqueous solutions. Meanwhile study on metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals by Mejare and Bulow (2001) focused on role of microorganism in enhancing heavy metal accumulation and restricted the scope on different peptides and proteins towards Cadmium (Cd II) [6]. The selection of skim serum as proteins source in this works is mainly to eliminate the long homogenization process commonly involved in protein purification and yet is found successful in collecting most of removed hevea proteins which is technically have other role to be revealed.

This researched been conducted to give some value added to hevea proteins which always been misunderstood as the only culprit in latex product industry as allergen and cause allergic reactions. This report were focused on comparing waste proteins extraction methods by investigating yield and physical characteristics. The extracted waste proteins then were used to bind metal of various types and concentration.

2. Material and methods

2.1 Material and instrument
Natural rubber latex skim wastewater was collected from MARDEC Industrial Latex Tapah Perak Malaysia. Ammonium sulphate granule of Qrec brand was purchased from TRP Technologies Malaysia. 3.5 kDa molecular weight cut off snake skin dialyzing tube was purchased from Research Instrument. Standard metal solution was purchased from MERCK Malaysia. Refrigerated centrifuge velocity 18R Dynamica was used in protein precipitation. FTIR–ATR Spectrum 400 from Perkin Elmer was used for the molecular study. Varian Cary 50 UV spectrophotometer of Anton Paar was used to offer indications of the presence of protein and protein concentrations.

2.2 Methodology
NRL skim waste collected and kept in chiller (4 to 11°C) was centrifuge at room temperature 14000 rpm speed for 15 min to expel rubber particle to the top. 25 ml of the middle layer of centrifuged NRL waste was pipetted and transferred to the conical flask for the salting out using saturated ammonium sulphate solution. The solution were then centrifuged at same rpm and speed to precipitate the protein. The supernatant was removed and the precipitates was allowed to dry and weighted. The precipitates were then re-dissolved using de-ionized water to 5 ml and transferred to 3.5 k Da molecular weight cut off snake skin dialyzing tube. The tube was soaked in de-ionized water and kept in chiller for 3 days to remove the remaining salt presence in the precipitated protein. After 3 days precipitated solution was then transferred to 25 ml Schott bottle for freeze drying process. Freeze drying required 1-2 weeks depends on the sample purity. Freeze dried proteins were then weighed and transferred to plastics petri dish for storage purposes and kept in - 20°C for freshness. Parameter varied for precipitation were pH media prior to precipitation and absence or presence of ammonium sulphate. For metal extraction analysis, freeze dried protein was weight for about 0.5 to 1 g and dissolved to 100 ml in volumetric flask. Metal solution were prepared from standard metal solution from 1000 ppm to 2, 5, 10, 15 and 20
ppm. 100 ml protein was divided into 5 portion with 20 ml each and mixed with each metal concentration. The blank reading was used as reference and final concentration of mixed protein with metal were used to calculate metal removal or metal that have been bound with that proteins. Parameter varied were metal solution concentration and volume of protein reacted with metal solution which were of 10 ml, 20 ml, 30 ml and 40 ml.

3. Result and discussion

Purification process were evaluated based on final dry amount of proteins collected. Three purification conditions; initial volume, dialyzing tube pore size and surrounding pH were compared. Purified and unpurified proteins were used for metal extraction study.

3.1 Effect of various purification steps on amount of protein collected

*Hevea* proteins collected contains several different physical characteristics thus varying some physical aids in extraction is expected to improve amount extracted. By varying pore size to the smallest for example more proteins were managed to be extracted. Different pH surrounding were introduce in order to influence basic or acidic proteins to be more pronounce and easily been extracted during purification.

| Tube MWCO K Da | Weight 1(g) | Weight 2(g) | Weight 3(g) | Average (g) |
|---------------|-------------|-------------|-------------|-------------|
| 3.5           | 0.2847      | 0.2993      | 0.3712      | 0.3184      |
| 20            | 0.1696      | 0.1773      | 0.1671      | 0.1713      |
| 40            | 0.1354      | 0.1597      | 0.1159      | 0.137       |

Effect of tube size was carried out to measure the amount of protein collected when different size used at a same starting volume and other parameter was kept constant. Result showed for 20 k MWCO the average amount of protein collected was reduced by 46% and for 40 k MWCO it was further reduced to 57% which was more than half. This lead to decision on using 3.5 k MWCO. The reason for this was the range of size of *hev* proteins. Theoretically NRL serum contain proteins ranges from 5 k Da to 50 k Da in molecular weight [7]. Besides that it was also possible to confine specific *hev* proteins base on size by that method but due to amount collected is also one of our main concern, other dialyzing tube size were not taken into consideration in this work. Different pH surrounding only provide slight increment in final amount of protein collected at pH 4, 6, 7 and 8 which supported previous works that reported the effect of pH on protein, which is extractable protein (EP) content of NR films decreases with decreasing pH, where the reduction of EP with increasing acid content is due to the denaturation of the proteins by acid [8]. In looking for the best dialyzing tube size (Table 1), the smaller size the better due to *hev* protein ranges from about 10 k Da to 200 k Da [9] that’s answer why the use of 40 k Da tube reduced the amount of protein collected by more than 50% as 3.5 k (0.3184 g) and 40 k (0.137 g) were conducted to investigate if there is any significant changes in amount of protein collected when NRL waste were exposed at different pH due to the fact that the identified 15 *hev* proteins have isoelectric points ranges from 3.5 to 9.5 [9].

It was wished that the active centre of specific *hev* protein will apparent but from the data obtained only at pH 5 the slight reduction in collected amount which might be due to some error in the experimental works, other than that not so significant to jump into conclusion in focused on specific pH condition. Previous works had reported that enhanced ionization of proteins and phospholipids at alkaline pH causes increase in negative charges of the latex particles that contributed to increase in stability which also answered why increment in amount of purified proteins at alkaline pH [10].
Table 2. Effect of pH on extracted proteins weight [measured after freeze drying]

| Precondition | Weight 1 | Weight 2 | Weight 3 | Average |
|--------------|----------|----------|----------|---------|
| DI water     | 0.2467   | 0.2454   | 0.2047   | 0.2322  |
| pH4          | 0.2885   | 0.2003   | 0.2844   | 0.2577  |
| pH5          | 0.1931   | 0.1719   | 0.1651   | 0.1767  |
| pH6          | 0.1905   | 0.2687   | 0.2992   | 0.2528  |
| pH7          | 0.223    | 0.211    | 0.3      | 0.2446  |
| pH8          | 0.2717   | 0.2610   | 0.3076   | 0.3011  |

3.2 FTIR study of purified protein

Fourier Transform infrared spectroscopy is well suited to detect relative changes in protein secondary structure due to external factors by analysing the amide I band of proteins between 1700 and 1600 cm^{-1} this band is influence by hydrogen bonds which are mainly effected during conformational changes in protein secondary structure [11]. Previous study on protein using FTIR listed that amide I peaks occur at 1630 cm^{-1}, Amide II at 1541 cm^{-1} and 3283 cm^{-1} (N-H) stretching [12]. From figure 1, frequency 1090 cm^{-1} was assigned to twisting –CH2- while 1446 cm^{-1} for –CH2- deformation. Only one sample of purified protein shows existence of Amide II. However a broad peak at 1638 cm^{-1} confirms the existence of protein but there is slight variation in intensity could be seen from the Amide I peak (stretching –(C=O) - NH-R). An intense broad band between 3200 and 3500 cm^{-1} indicating the presence of hydroxyl groups [13] thus (N-H) stretching peak at 3283 cm^{-1} is expected to overlap with hydroxyl peak because this protein is dissolved in water, furthermore this 3280 cm^{-1} peak more apparent in cream rubber but our sample is the skim rubber that result in this region will not be taken into consideration for this study. It can be seen that all purified proteins exhibit higher intensity due to purification removes or reduces all unnecessary peaks. From waste water hevea proteins were extracted by a series of extraction process so that answer why they showed clearer or increase in specific concentration of said structures.

![Figure 1](image-url)  
Figure 1. FTIR spectrum for NRL serum (A), purified protein using 4.1 M ammonium sulphate solution (B) (purified protein 1) and pallet ammonium sulphate (C) (purified protein 2)

Figure 2 comparing FTIR of purified protein at frequency 1000-1200 cm^{-1}, 1600 to 1500 cm^{-1} and 1600-1700 cm^{-1} which were assigned for –CH2-, Amide I and Amide II large variation displayed by CH2 peak meanwhile for amide I and amide II less than 2% at every point were observed.
3.3 Metal extraction of NRL waste, MC purified and SP purified protein

Metal extraction was calculated from percent loss when standard metal solutions of multi elements were reacted with protein solution at various concentration. Multi element metal solution were prepared from 2 ppm to 20 ppm concentration and result tabulated were at 5 ppm concentration reacted with unpurified to different type of purifications. Table 3 showed metal removal in percentage at 5 ppm metal concentration of mix Pb, Cu, Cd, Al and Zn.. The metal species removed remain 4 and Zinc still cannot be removed. However The above samples were qualitatively evaluated for metal concentration using FTIR spectroscopy. At lowest protein concentration (5:10), percent metal removal is around 20 to 30%, as the concentration increased the percent also increased due to metal had bound with proteins therein. The consistent increment were exhibits disregard of purification level and metal species except for Zn which cannot tolerated with this *hevea* proteins due to Zn itself is presence in the NRL waste and there is no empty spaces for Zn to bind with those proteins.

| Concentration metal: proteins | NRL/ MC/ SP Pb removal % | NRL/ MC/ SP Cu removal % | NRL/MC/SP Cd removal % | NRL/MC/SP Al removal % | NRL/MC/SP Zn removal % |
|-------------------------------|---------------------------|---------------------------|------------------------|------------------------|------------------------|
| 5:10                          | 34.2/20.2/28.6            | 14.2/14.9/26.5            | 22.4/18.9/27.6         | 8.3/20.3/30.1          | -2.9/19.2              |
| 5:20                          | 56.4/28.6/38.1            | 3.0/26.7/35.9             | 18.3/29.2/36.7         | 1.8/32.8/38.5          | -5.5/37.4              |
| 5:30                          | 64.3/48.5/51.0            | 24.3/47.3/48.9            | 51.7/46.3/50.7         | 27.8/44.9/51           | -4.2/48.2              |
| 5:40                          | 61.1/49.7/60.4            | 26.0/45.9/58              | 45.3/47.3/61.8         | 32.4/46.9/60.2         | -5.1/36.7              |

Note: NRL-NRL waste, MC –multiple centrifuge, SP- ammonium sulphate purified

FTIR spectrums exhibited fairly consistent with the concentration of proteins measured using ICP. For figure 3 (a) maximum intensity in transmittance was achieved at 5 ppm metal with 40 ml protein solution, followed by 5:20, 5:30 and 5:10 the 5.30 peak which is supposed to be second may be due to impurities which is still present in filtered NRL waste. Figure 3 (b) was a MC purified proteins , the 5:40 peak is at highest intensity followed by 5:20 and 5:30 at the same position and lowest intensity was at 5:10. This shows the correct trend for proportional concept as the concentration increase the intensity also in elevation. Figure 3 (c) is the SP purified protein which also gave slight different trend in concentration order where 5:20 showed very weak intensity compared to 5:10 but in terms of total intensity different SP purified beat all other proteins specimens. The consistent pattern displayed in FTIR spectrum and calculation of metal removed from ICP indicating possible rapid determination or presence of metal protein by FTIR instruments [14].

![Figure 2: MC purified and SP purified proteins](image-url)
3.4 FTIR spectrum of metal extracted solution.
Spectroscopic method was rapid and relatively easy to use but it depends on light absorbance transmittance and may be compromised by opaque or coloured solutions [15]. Comparing image in figure 1, 2 and 3, it can be seen that from purification proteins region at amide I and amide II does not displayed much different, however at region 900-1200 cm\(^{-1}\), significant different in the spectrum can be viewed but this 900-1200 cm\(^{-1}\) was assigned to \(-\text{CH}_2\). Other peaks that are not associated to isoprene are an intense band between 3200 and 3500 cm\(^{-1}\) indicating the presence of hydroxyl groups, an amide (N-H) peak at 3280 cm\(^{-1}\) (more apparent in cream rubber), a carbonyl peak (C-O) peak at 1737 cm\(^{-1}\), a small amide (N-H) peak at 1548 cm\(^{-1}\) (both more apparent in cream rubber) and a series of peaks between 1130 and 1010 cm\(^{-1}\) indicating oxygenated compounds.

![FTIR spectrum of metal extracted solution](image)

**Figure 3.** FTIR spectrum of metal bind NRL waste (a), MC protein (b) and SP protein (c)

*meX5:10*  *meX5:20*  *meX5:30*  *meX5:40*

The peak at around 1080 cm\(^{-1}\) is assigned to the (C-O) groups [13] which answer the significant difference in concentration when those protein reacted with metal changed in oxygenated compounds concentration. From figure 3 a), b) and c) it can be seen that there is only little variation in pattern for region 1600-1700 cm\(^{-1}\) and 1500 to 1600 cm\(^{-1}\) which are amide I and amide II but from 900-1300 cm\(^{-1}\) shows a huge variation in peak obtained. The consistent form displayed engaged the investigation on metal extraction sample (4) and the relations on the 900-1200 cm\(^{-1}\) region.
4. Conclusion
Purification parameters study were conducted in a seek of basics knowledge of NRL waste and factors contributing to the final purified amount. Standard purification (SP) is best purification method of NRL waste protein but MC method still useful due to the advantage of no chemical were used and less processing step thus less cost incurred. On the other hands purification condition using different pH surrounding may require a few more steps to confirm specific pH condition that best suited targeted proteins. For instance FTIR can be used as a rapid method to investigate presence of purified proteins but a set of established spectrums may be necessary to confirm protein concentration.

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