International Journal of Engineering & Technology, 7 (4.14) (2018) 426-429

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper



Metal Selectivity of Hevea Protein Isolated from Natural Rubber Latex Skim Serum

H. Tahir[1], M. Misran[2], M.R. Othman[1] and N. Ibrahim[1]

[1]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.
[2]Faculty of Sciences, University of Malaya, Malaysia
**Corresponding author E-mail: haira331@salam.uitm.edu.my*

Abstract

Hevea protein isolated from skim serum, a by-product of centrifugation process, contains useful proteins in extracting metal. It can be used directly from the source or purified before reacting with metal solutions. Those proteins bind with metal at varying degrees. Upon exposure from as low as 2 ppm concentration to up to 20 ppm metal solution concentration, different binding characteristics were seen. The reasons of such inconsistency in the characteristics might be due to the existence of some of the metal itself in the NRL serum. Mg^{++} and Zn^{++} are common metal found in NRL products and those metals would show the slightest in binding with *hevea protein.* Other metals which were covered in this scope of study shows a good binding characteristics disregard of the group of metals belongs. Selectivity was measured from the final concentration of metal in percentage. In most cases, lead, copper and cadmium show good interacti on with *hevea proteins*.

Keywords: *Natural rubber latex, Hevea protein, metal selectivity*

1. Introduction

Purified protein from skim serum of NR latex concentrate protein contains *hev* proteins with various biological roles. Metal cations are indispensable components of the cellular machinery and involve in numerous essential tasks, ranging from nucleic acid and protein structure stabilization to enzyme catalysis, signal transduction, muscle contraction, hormone secretion, taste and pain sensation respiration and photosynthesis [1]. Metal ion are required for the growth of life forms, about half of all proteins contain metal ions and metal ions perform a wide variety of specific functions associated with life processes [2]. Organism responds to heavy metals stress using different defense system, such as exclusion, compartmentalization, making complexes and the synthesis of binding proteins such as *metallothioneins (MTs) or phytochelatins (PCs*) [3]. Heavy metal ion presents in the soil can be taken up alongside nutrients with water and incorporated into plant tissues. Metal and metalloids that are toxic to plants include copper, iron, manganese, zinc, nickel, chromium, aluminum, cobalt, cadmium, molybdenum, arsenic and lead, but in the advent of excessive heavy metal toxicities, susceptible plants rapidly initiate defense mechanism [4]. Plant defense responses are thus required to protect cell and subcellular compartments against oxidative damage [5]. In metal coordination chemistry, the term complex means a central metal atom or ions surrounded by a set of ligands, a large number of molecules and ions can behave as ligands, and a large number of metals ions form complexes [6]. A number of functional groups participate in metal binding in metalloproteins; the side chains of Glu , Tyr, Cys, His Arg, Lys, Asp and Met and although free cysteines can potentially bind chelated metals, in practice they are rarely available in the appropriate reduced state [7].In new dimension of protein separations, the apparent affinity of a protein for a metal chelate depends strongly on the metal ion involved in coordination. Protein retention on different metal mirrors the affinity of the metal for imidazole. The stability constant for complexation with imidazole follows the order $Cu^{2+} > Zn^{2+}$ and also depends on the quantity of protein that can be loaded onto a given metal-chelate support [7]. Immobilized metal ion chromatography (IMAC) was used to purify protein by using metal binding concept, Ueda *et al.*, (2003). Binding of proteins (or peptides) to metal ions is based on the interaction between an electron donating group present on a protein surface and a metal ion presenting one or more accessible coordination sites. In IMAC, use is made of a sorbent, or matrix, to which metal-chelating groups are covalently attached. When metal ion were added (loaded), the multidentate chelators and metal ion form complexes in which alkylation by phenyl transfer in enzyme catalyzed reaction. The effect of Mg^{2+}, Mn^{2+}, Ca^{2+} and Cu^{2+} on rubber transferase activity tested had found only Mg^{2+} and Mn^{2+} were activating while Cu^{2+} were inhibitor [4]. Guo *et al*.,(2014), in their research work on binding between lead ions and the high-abundance serum protein, discussed on the interaction between three of the most abundant bovine serum proteins (serum albumin, transferrin and IgG with Pb^{2+}) using electrochemistry. The binding constant (β) of Pb^{2+} to the individual proteins and a mixture of proteins were measured according to non-ideal state theoretical equation as well as McGhee-Von Hippel equation (Ideal state). Protein-protein interactions and micro-environmental influences the binding between Pb^{2+} and serum protein [8]. In this work, the focused were to evaluate binding capability of different metals towards NRL waste proteins extractions. NRL waste without purified were used as basis to purification via standard ammonium salt purification method and physical method by using centrifuge or preconcentration method in comparing metal selectivity. Protein dried amount was measured to have a clear view of effect of specific pH toward final purified amount. Two approach were conducted to

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investigate metal activity i. Purified and unpurified proteins towards different metal concentration and ii. Purified via SP and MC at specific pHs. The finding was used in evaluating metal selectivity based on percentage of metal removed.

2. Material and methods

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 k Da 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 and UV Vis CARY were used for the concentration and molecular study. Freeze dried protein was weighed 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. Initial metal concentration in the NRL serum was measured and used as a guideline in estimating *hevea protein* metal selectivity. Each pH of mix NRL proteins and metal solution were recorded to investigate the general effect of pH on percentage metal removal and selectivity.

3. Result and Discussion

Table 1 showed protein concentration measured as a result of varying the pH surrounding. The finding proved that specific pH gave different purified amount suggesting that *hevea* proteins are pH dependent protein thus purification via pH gradient might be used in future work.

Table 1: Protein concentration measured using Bradford assay and resulting dried weight

Sample	Protein concentration µg/ml	Protein dried weight g
NRL waste	608	-
pH4 MC	729	0.187
pH5 MC	786	0.193
pH6 MC	747	0.19
pH7 MC	764	0.179
pH8 MC	730	0.166
pH4 SP	1044	0.288
pH5 SP	828	0.465
pH6 SP	885	0.268
pH7 SP	691	0.3
pH8 SP	738	0.261

3.1 Metal extraction analysis for NRL waste proteins

NRL waste collected from latex concentrate centrifuge process was filtered before introducing metal element to study the binding characteristics of such free protein inside without purification was found acceptable when exposing up to 10 ppm of multi elements. NRL waste serum was filtered using filter paper for a few times and analysed for traces of metal using ICP. It was found that only Zn^{2+} have 254 ppm and 19.7 ppm $Mg^{2+.}$ For lead, cadmium and copper and aurum there were no traces that could be measured as interference in this study. Study on metal cofactors which investigated metal content in NRL found that about 12.04 mM or equivalent to 1204 ppm Mg 2+, 25.94 µM of Cu2+ or equivalent to 25.94 ppm and 413.82 µM of Zn 2+ or equivalent to 413.82 ppm [4]. Comparing those values and the data collected from NRL waste of skim rubber production, there was a major reduction in Mg^{2+} concentration but only about 50 percent reduction in concentration of Zn^{2+}. The selection of skim serum waste as a starting material for the protein purification is a correct choice because the skim did not only retain high protein concentration but also reduced concentration of metal that made possible for the skim serum to be used in metal removal because it will allow more spaces for metal binding.

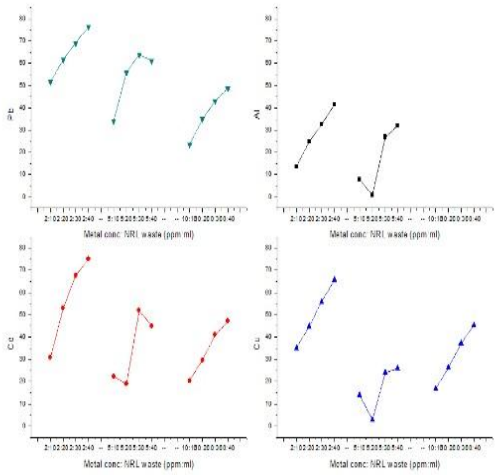

Fig 1: Effect of NRL waste protein concentration on metal selectivity

Note: at 2 ppm (i) and 5 ppm (ii) metal solution concentration
Note: pH for 2:10, 2:20, 2:30, 2:40 were 2.63, 2.73, 2.78, 2.86, 5:10,5:20,5:30 and5:40 were 2.20, 2.26, 2.36, 2.46, 10:10,10:20,10:30,10:40 were 1.97, 2.02, 2.14, 2.25 accordingly

The selection of metal bound to protein is of best interest in this work to construct *hevea protein* metal interaction model. From figure 1 it can be seen that out of 5 metals reacted with NRL waste, only 4 managed to be removed for up to 70% namely Pb, Cd, Al and Cu. The selection of metal was determined by the end terminal of free protein and yet in this works Pb^{++} seems to be the most common metal to be bound with. Meanwhile Zn^{++} which is one of the common elements inside the NR waste cannot be removed even if it is at only 2 ppm concentration. In interaction between Zn^{++} and Cu^{++} ions on protein surfaces, imidazolyl, thiol and indolyl functional groups are the main targets for the metal ions [9]. Cadmium is the second element in the list of selected metal bound with *hevea* protein followed by copper and aluminum for 5 ppm metal concentration but at 10 ppm only lead, cadmium and copper could be tolerated by *hevea* protein. Aluminum is biochemically reactive. There are no ligands or chaperons which are specific to its transport and no transporters or channels to selectively facilitate its passage across membrane. There are also no intracellular storage proteins to aid its cellular homeostasis and no pathways which evolved to enable the metabolism and excretion of aluminum [10]. Lead is one of the main sources of pollution in the environment and is severely toxic to plants and animal. It interferes with the physiology and metabolism of the plants by binding to the sulfhydryl groups of various proteins, leading to structure disruption or activity inhibition [11]. On the other hand, multiple centrifuge purified hevea protein fully utilized the centrifugal force and difference in specific gravity of different molecules as separating tools. This method was expected to works well in the system which the surrounding media and protein have significant difference in specific gravity but somehow the external factor such as surrounding pH is an added advantage for a range of pI of hevea proteins in the NRL waste.

3.2 Metal extraction analysis of multiple centrifuges (MC) purified proteins

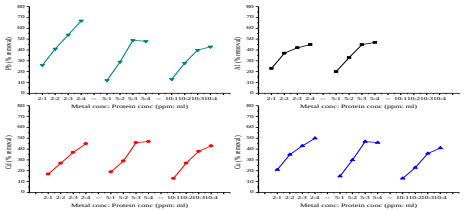

Fig 2: Effect of MC centrifuge protein concentration on metal selectivity 2 ppm, 5 ppm and at 10 ppm metal solution concentration
Note; pH for 2:10, 2:20, 2:30,2:40 were 4.73, 5.05,5.10,5.19, pH for 5:10 , 5:20, 5:30, 5:40 were 2.16,2.21,2.30,2.36 and pH for 10:10,10:20,10:30,10:40 were 3.85, 4.55, 4.78, 4.91 accordingly

Multiple centrifuge purified proteins are purified protein which make use of centrifugal force and density concept to float the protein, concentrate the protein in the waste and use dialysed tube to confine specific molecular weight protein and freeze dried it. Purified protein of this methods largely depends on the technique and concentration of protein in the initial waste. From figure 3, prevalent metal still leads for all metal concentration which exactly followed the pattern of NRL waste binding characteristics. Except that for all metals concentration different type of metal does not gave much different on removal compared to NRL waste. This is due to purification that confine the protein capability of removing metal at equal balance. From figure 3, significant difference in capability of removing metal is around 60 % for lead and aluminum. Meanwhile for MC purified protein, there was only around 40% difference. Similar pattern of MC is displayed for NRL waste on the metal selected and % removal. However, the removal line constructed is consistent with SP which are at 5 ppm metal concentration. MC cannot tolerate 20 ml proteins but is good at 30 and 40 ml. These occurrence is may be due to MC had achieved certain degree of purification but salt (SP) did better in protein purification.

3.3 Metal extraction analysis of ammonium sulphate purified (SP) proteins

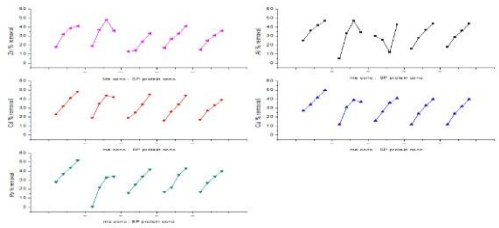

Figure 2: (i) Effect of concentration of (SP) protein on metal removal from left
Note at (i) 2 ppm (ii) 5 ppm iii) 10ppm iv) 15ppm and v) 20ppm metal solution concentration
Note pH for 2:10,2:20,2:30,2:40 were 2.29,2.43,2.65,2.85 ,pH for 5:10,5:20,5:30,5:40,2.16,2.31,2.45,2.47,pH10:10,10:20,10:30, 10:40 1.95, 2.06, 2.10,2.21, pH for 15:10, 15:20 15:30, 15:40 1.52, 1.62, 1.68, 1.76 and 20:10, 20:20, 20:30, 20:40 1.74, 1.76, 1.82, 1.84 accordingly

SP purified protein can tolerate five elements when reacted with metal concentration from 2 ppm, 5 ppm, 10 ppm,15 ppm and 20 ppm and still have not shown any reduction in metal removal capability. The interaction of proteins of metals here were not affected by any disturbances from other impurities presence in NRL waste and MC purified. This salt purified protein had achieved acceptable degree of purity and caused consistency in metal removal of all metal concentration. Metal concentration was reacted to protein at up to 20 ppm and the trend in removal still increased. Metal removal of ammonium sulphate purified *hevea protein* were also investigated to find the selectivity of metal towards hevea proteins and it was found that the pattern changes with metal concentration. As the concentration of metal increased metal removed were in order of Pb > Zn > Cd > Al. At lower metal concentration lead still dominated the binding species but as the metal concentration increased the selectivity changed to Zn^{++} at 5 ppm, Cd^{++} at 10 and 15 ppm and Aluminum at 20 ppm. Selection of metal bound protein now depends on concentration of metal or the surrounding pH because at higher concentration pH is at lowest or acidic. At either high or low extremes of pH , at least some of the charges on the protein are missing, so the electrostatic interactions that would normally stabilized the native protein are drastically reduced [12]. Previous works on Cd found that heat treatment which disrupted the cell of LAB absorbed more Cd (II) at pH 5 and pH 7 because heating disrupted the bacterial cell so that intracellular proteins may bind the Cd (II) [13]. Comparing NRL waste, MC and SP purified proteins, only SP can tolerate Zinc ions which is already exist in NRL waste and still exist at certain concentration in MC purified which cause the limitation in tolerating induced Zinc. Zinc is an essential element in biology and it has several properties that distinguish it from other transition elements; it is a strong Lewis acid with no redox roles under physiological conditions, but serves as cofactor for a number of enzymes. Furthermore, zinc chelate complexes tend to be thermodynamically stable, so zinc can serve as a scaffold that maintain the proper folded structure of proteins. However at high concentrations zinc is toxic [14].

3.4 Metal extraction of different pH multiple centrifuge (MC) prepared proteins compared with Standard purified (SP) prepared protein

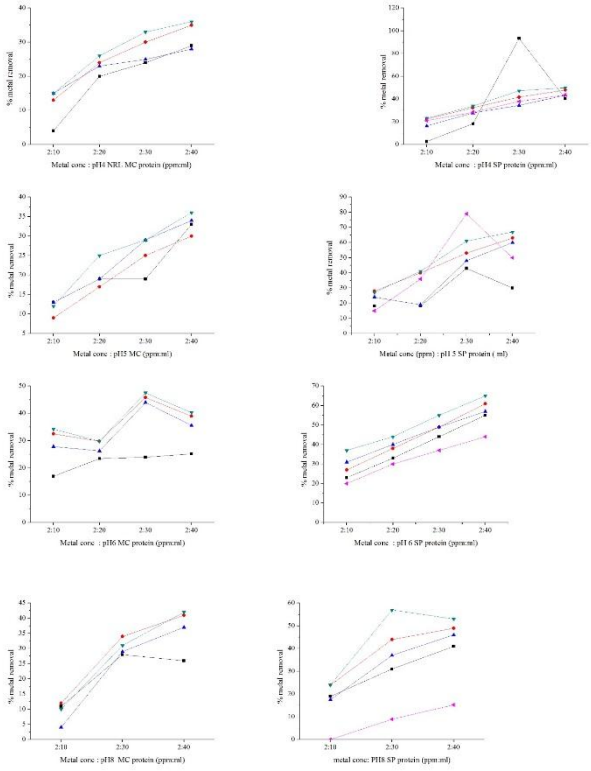

Figure 4: Effect of concentration of pH on metal removal (i) pH 4(MC) and (ii) pH 4(SP) protein on metal removal at 2 ppm metal solution concentration of (iii) pH5 (MC) protein and (iv) pH5 (SP) on metal removal at 2 ppm metal solution concentration,(v) pH 6 (MC) and (vi) pH 6 (SP) protein on metal removal at 2 ppm metal solution concentration (vii) pH 8 MC and (viii) pH 8 SP protein on metal removal at 2 ppm metal solution concentration

Note: pH of pH4 SP 2:10.2:20,2:30,2:40 were 2.95, 3.33,4.07,5.57,pH6 MC 2:10,2:20,2:30,2:40 were 2.88,3.04,3.20,3.45, pH7 SP 2:10,2:20,2:30 were 6.94,6.87,6.81, pH8 MC 2:10,2:20,2:30 were 2.81,3.14,3.35, pH8 SP 2:10,2:20,2:30 were 2.97,6.07,6.46 accordingly.

Variation in surrounding pH was implemented in purifying NRL proteins as an attempt either to increase purification amount or capture specific proteins so that maximum potential application is achieved. Unfortunately, both objectives were not successfully driven. This is may be due to selection of pH or other purification steps that limit the isolation of specific proteins and also there is no significant increase in purified quantity. Comparing pH purified sample from MC and SP on metal extraction capability, the plotted pattern remain the same as the other type of protein samples, where MC purified can only tolerate four types of metal while SP purified can tolerate all metals despite of the surrounding pH used at the same 2 % metal concentration. From figure 4 (i) and (ii), closed gap between each metal removal represent SP purified while for MC purified a distance in metal removal capability gave the same pattern as using de-ionized alone on purifying the proteins. Even though the close and distance gap were shown, the maximum percentage removal remained close with lead still be the most chosen metal to be removed. Consistent profile in selection of metal removal was showed by MC purified protein from pH 4 to pH 8 purified condition. Percentage removal does not show any significant difference except at pH 6 and pH 8 with slight increment in percentage removal. However, at pH 6 purified protein the maximum removal fall at 30 ml proteins and other pH showed increase in percent removal. This trend also occurred for SP purified protein at 30 ml proteins whereby at pH 4 for aluminum and at pH 5 for aluminum and zinc started to decrease at that point. The percentage removal showed proportional reduction from pH4 to pH8 for SP purified with reduction about 20 percent at every pH difference. The reason of reduction in percent removal might be related to the pH of the protein that influenced metal removal where alkali pH does not promote metal binding. Previous study had indicated that histidine is the amino acid with the strongest affinity for metal ions and it was widely accepted that histidine, tryptophan and cysteine residue are a key player in the binding of protein in IMAC [9].

4. Conclusion

SP purified protein use ammonium salt to remove water bound layer surrounding the protein that cause aggregation and precipitation of proteins. MC purified protein is a physical method which only use centrifugal force which largely depends on speed, angle and density of proteins to the surrounding water layer that holds the protein. Speed of 14000 rpm that have been used in this research only cause partial purification of protein which holds greater difference in density with that surrounding layer. Other proteins might be lost during the purification thus result in the same capability as NRL waste with only slight consistent in removal trends. The reason for NRL waste could tolerate certain concentration and specific element is because of the impurity in the waste does not cause disturbance in metal removal but the removal may not only cause by the proteins inside the waste which is not purified yet. Other reasons of inconsistency or selection of elements are might be due to the degree of interaction of protein to metal and the chances of unpurified protein particle to meet the metal ions which will contribute to the removal profile. Overall profile displayed that lead, Pb is easily bound with heavy metal SP protein, MC protein or unpurified (NRL waste). Order of metal selectivity for NRL waste is Pb> Cd>Cu>Al, for MC protein is Pb>Cu>Cd>Al and for SP more element were removed which is Pb>Cu>Zn>Cd>Al. Although the retention of proteins is primarily due to the metal affinities of the individual amino acids, other factors also contribute profoundly towards their metal affinity including amino acids sequences, folding and surface properties [9] .

Acknowledgement

The authors would like to thank the Research Management Institute (RMI), Universiti Teknologi MARA (UiTM) to finance the project under the Lestari Fund 600-IRMI/MyRA 5/3/LESTARI (101/2017) and Colloid Lab, Faculty of Science Univesiti Malaysa.

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