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Milk dependent hemagglutination inhibition and calcium alginate immobilization of ricin isolated from *Ricinus communis* L. seeds

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Abstract

Ricin, a lethal lectin is reported to be used in the purification techniques of proteins and cell surface glycoconjugates by immobilizing them onto the matrices of polymers. Ricin can also bind the glycoproteins present on the membranes of Red Blood Cells (RBCs) resulting in visible agglutination. They are the major phytotoxins of castor beans (Ricinus communis L.) from where they were isolated using 60% ammonium sulphate precipitation in our study. The partially purified form of the isolated ricin was characterized for temperature stability by Tube Hemagglutination Test (THAT) and Milk dependent hemagglutination inhibition by Slide Hemagglutination Test (SHAT) along with immobilization with calcium alginate gel beads. THAT, SHAT and immobilized activities were tested on 3% RBC suspension which showed the efficiency of the extracted ricin. The maximum temperature stability with significant reduction in the agglutination was recorded at 80℃ and at 95℃, no clumping activity was noted. The results obtained from SHAT infer that ricin is inhibited by the increasing concentrations of milk. At the initial concentration of milk in the mixture (0.275%), hemagglutination was comparatively seen higher and at 0.625%, ricin was completely inhibited by the milk. The immobilized calcium alginate-ricin beads were crushed to expose the entrapped lectins to the RBCs and the interaction was checked microscopically. Immobilized ricin could potentially bind the RBCs surrounding the slurry and was confirmed upon microscopic evaluation.
Keywords: Ricin, Temperature stability, Red Blood Cells, Milk, Slide Hemagglutination Test, Calcium alginate immobilization

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1 Introduction

Lectins are proteins which have been widely used to study about glycoproteins, glycolipids and other biomolecules. These are almost present in every plant which is helpful for their defense mechanisms; also, lectins play a vital role in signal transmission and immunological responses in higher organisms (Blot et al., 2022). Lectins can bind specifically and interact with the molecules present on the surfaces of the cells such as Red Blood Cells (RBC) (Sandvig et al., 1976), endothelial cells, tumor cells and microorganisms causing visible agglutination (Sharon and Lis, 2004). Ricin and *Ricinus communis* Agglutinin (RCA) are the two major lectins present in the seeds of castor (Rana et al., 2012). Ricin is a toxin that creates a negative effect in the protein synthesis of most eukaryotic cells by inhibiting the translation process (Lord et al., 1994). The biological function of RCA is that it binds with most of the cells in our body preferably vascular endothelial cells. Comparatively, RCA is less toxic than that of ricin but it is the highly homologous protein present in *Ricinus communis* L. plants (Sandvig and Deurs, 2000). Ricin is the major constituent of castor seeds and has a very high toxicity which might be lethal. Regular consumption of these seeds or oil as food sources without proper treatments for protein denaturation might lead to slow poisoning (Purkait and Koley, 2019). Hence, it has become important to study the effects of lectins like ricin on biological systems, develop practical methods and find cure for the disease.

Ricin can be inhibited by most of the molecules present in the food we consume daily. To support this statement, Youle et al. 1981 showed that ricin has galactose and lactose binding domains in its structure; thus, lactose and galactose can be used to block the activity of ricin on eukaryotic ribosome (Olsnes and Pihl, 1972). So, such kinds of interactions by these molecules with ricin can also reduce the interaction of lectins with receptors of the cells present in our body. Rasooly et al. 2012 reported the inhibition studies of ricin by milk at in vitro conditions on vero cells which are cell lines derived from the kidney of an African green monkey. These evidences strongly support the treatment remedies at several circumstances for ricin toxicity. The toxicity of ricin has been dealt in many literatures based on the haemagglutination property especially using Red Blood Cells which pave way for a precise conclusion. Although, this article aims to purify ricin from the castor beans and characterize the effects of ricin based on the temperature stability by following the usual techniques. Also, inhibition studies have been performed in the present study suggesting that milk could be a potent source for reducing the toxicity of ricin in the body.

Immobilization is a technique used for trapping proteins, enzymes, microbes and cells without losing their metabolic properties. This leads to advancements in the fields of analytical chemistry, biological oriented industries and clinical studies (Rao et al., 1998). Immobilization of proteins in the form of calcium alginate beads is one of the finest methods for researchers to adapt. Calcium alginate is a polymer that has the ability to ideally entrap the other molecules with minimal drawbacks during practical applications. Calcium alginate beads are made by mixing the desired concentrations of sodium alginate and calcium chloride solutions along with the biological mixture to be entrapped (Sharmila et al., 2012). This allows the calcium ions to cross link the polymers of alginate forming spherical, soft and intact gel beads and the same procedure was trailed in our research for studying the immobilized activity of ricin on RBCs.

2 Materials and Methods

Materials

Sodium alginate, sodium chloride, calcium chloride and ammonium sulphate used for the exper-

imental study were of analytical grades. Castor (*Ricinus communis* L.) seeds and fat-free milk powder were purchased from a local grocery store in Coimbatore. Phosphate Buffered Saline (PBS) was prepared freshly and the pH was adjusted to 7.0 prior to lectin extraction.

Isolation of Ricin

Ricin was isolated by salting out the seed extract with ammonium sulphate (Trung et al., 2016) and dialyzed the pellet using membrane dialysis tube. *Ricinus communis* L. seeds were pre-soaked in distilled water overnight at room temperature for the ease of removal of seed coats. After seed coat removal, 1g of seed was ground using mortar and pestle in 50mL of PBS. The slurry was collected and transferred to sterile centrifuge tubes and incubated at 4°C overnight. After incubation, the tubes were centrifuged at 10,000x g for 15 minutes at 4°C. The supernatants were collected from all the tubes which contain crude lectin extract. To this extract, 60% saturated ammonium sulphate was added and centrifuged at 10,000x g for 20 minutes at 4°C. The precipitate obtained was dissolved in PBS and dialysed against 1000mL of distilled water (Shinde and Soni 2014) for three consecutive changes every 8 hours. The dialysate contains the partially purified ricin from *Ricinus communis* L. and was used for further experimental studies.

Preparation of 3% RBC suspension and temperature stability characterization

2mL of blood from a healthy donor with informed consent was collected in an EDTA tube. The collected blood was spun at 5000 rpm for 20 minutes. The plasma was removed and the resultant RBC pellet was washed twice with 0.9% sodium chloride solution and diluted to 3% RBC suspension (Jawade et al., 2016).

1mL of the partially purified lectin samples were taken in five different micro centrifuge tubes. The tubes were maintained at 28°C, 37°C, 55°C, 80°C and 95°C respectively for 30 minutes. Tube Hemagglutination Test (THAT) was performed to determine the temperature stability of the isolated lectin. Briefly, 700μL of the lectin mixtures incubated at varying temperatures were taken in test tubes and 300μL of 3% RBC suspension was added to each tube. The mixtures were left undisturbed for 30 minutes and clumping was noted.

Milk dependent hemagglutination inhibition assay

This technique is based on our own protocol. Varying concentrations (0.25%, 0.5%, 0.75%, 1.0% and 1.25%) of fat-free milk powder were prepared in distilled water. 1mL of the above solutions with different concentrations were dispensed into the respective tubes along with 1mL of ricin giving the final concentrations as 0.125%, 0.25%, 0.375%, 0.5% and 0.625% respectively. The tubes were incubated at RT for 20 minutes. Slide Hemagglutination Test (SHAT) was performed by taking 100μL from each test sample and added with 50 μL of the freshly prepared 3% RBC suspension onto microscope slides. SHAT for each sample was performed in duplicates. 100μL of 0.5% milk solution and 50μL of RBC solution served as negative control whereas, 100μL of pure ricin solution and 50μL of RBC solution served as positive control. Scoring was done as mentioned by Danielsson and Kronvall, 1974.

Immobilization of ricin

The extracted lectin from *Ricinus communis* L. was mixed with 10% sodium alginate solution

to yield a final concentration of about 4% sodium alginate-lectin mixture. Small drops of this mixture were dropped slowly into 0.4M calcium chloride solution (Mahajan et al., 2010). The beads were then collected by filtration.

Analysis of the immobilized ricin

The immobilized lectin-calcium alginate beads were partially dried by placing them on a filter paper. The beads were crushed finely to make slurry 0.2g of this slurry was added to 2mL of 3% RBC suspension and left at RT for 20 minutes. After incubation, the supernatant was decanted carefully leaving the sediment and washed with 0.9% sodium chloride solution. A small amount of the sediment was mounted with glycerol and examined microscopically.

3 Results and Discussion

Isolation of ricin

Partially purified lectin was isolated from the seeds of *Ricinus communis* L., Upon addition of saturated ammonium sulphate solution to the crude extract, a white fluffy precipitate of proteins was obtained. After subjecting this precipitate to dialyzation, most of the ammonium sulphate molecules bound to the lectins and other small molecular contaminants were removed thus, yielding partially purified ricin in high concentration. As most of the lectin molecules being globular proteins in nature, they can be effectively salted-out using ammonium sulphate by preventing the interaction with water molecules. In a literature, jacalin was extracted from jack fruit seeds and precipitated with ammonium sulphate and dialyzed against distilled water at 4℃ for 6h to remove the salts (Reddy et al., 2016). A lectin from Egyptian *Jatropha curcas* seeds was partially purified in the same manner of precipitation followed by dialyzation and the resultant dialysate was used for its characterization (Al-Saman et al., 2015).

Temperature stability characterization

The results are discussed in Figure 1. This experimental study reports that the isolated ricin from the castor seeds showed temperature stability up to 80℃ and at 95℃, the proteins may get denatured thus, losing their activity (Wood et al., 2018). However, the hemagglutination activity of the lectins may vary partially with respect to the purity of lectins, incubation period, RBCs collected from different individuals and other experimental conditions but increase when optimal temperature is reached and eventually decrease thereafter. In this regard, a lectin called abrin isolated from *Abrus precatorius* seeds showed its bioactivity till a maximum temperature of up to 74℃ (Banger et al., 2019). Similarly, *Phaseolus vulgaris* leucoagglutinin was thermally stable even more than 100℃ at pH 7.2 (Biswas and Kayastha, 2002). The biological activity of a lectin from *Moringa pterygosperma* decreased when the temperature exceeded 60℃ (De Mesa et al., 2004).

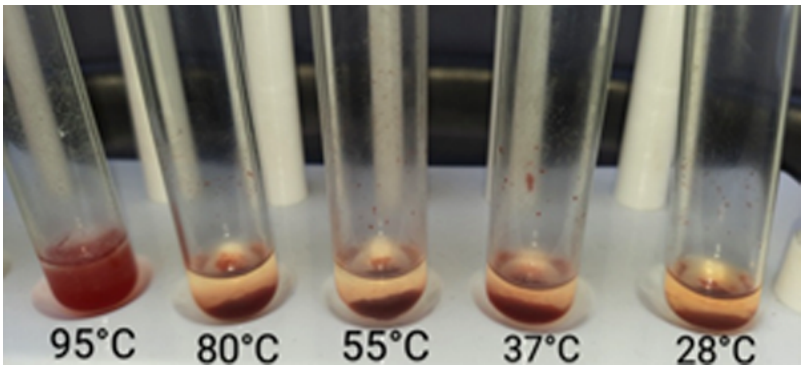

Figure 1. Tube Hemagglutination Test (THAT).

Milk dependent hemagglutination inhibition assay

Figure 2 discusses the Slide Hemagglutination Test (SHAT) which shows the activity of the extracted ricin decreased at increasing concentrations of milk in the mixtures. From the Table 1, it can be inferred that hemagglutination capabilities at 0.125% and 0.25% concentrations were significantly higher than other samples. Both 0.375% and 0.5% of milk affected the activity of ricin in a similar way but at 0.675% of milk concentration, the agglutination is completely lost. Milk contains lactose and galactose which bind to the Ricin-B chain and acts as a competitive inhibitor (Rasooly et al., 2012) against the surface glycoproteins of RBC membranes. Hence, this result concludes that milk is a potential inhibitor of ricin. N-acetyl glucosamine (NAG) and D-galactose both inhibited the hemagglutination activity of *Soybean lectins* (SBL) isolated from Glycine max on human erythrocytes at 0.01 mM concentration each (Bashir et al., 2010). The same NAG and D-galactose domains are present in the ricin structure as well. Similarly, the anti-H lectins extracted from the seeds of *Momordica dioica* was inhibited by the milk from human individuals (Joshi et al., 2005).

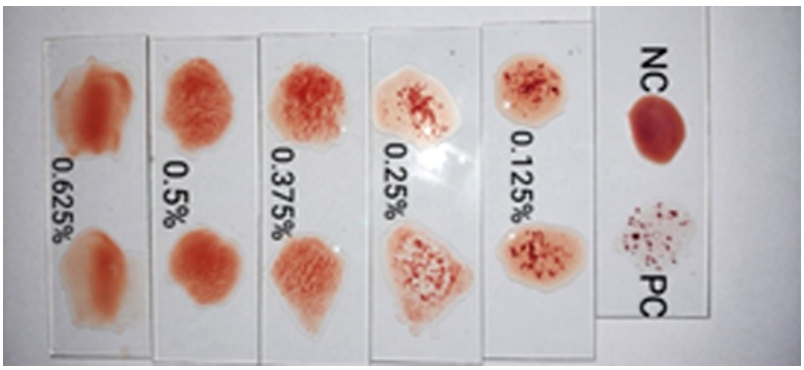

Figure 2. Slide Hemagglutination Test (SHAT) using different concentrations of milk in the sample.

Immobilization of ricin

Calcium alginate beads containing ricin extracted from *Ricinus communis* L. were produced by mixing 4% sodium alginate-lectin mixture and 0.4M calcium chloride solution. The beads consisting of the immobilized lectin synthesized as described above were found to be more intact which can withstand the physical conditions. In this regard, a literature mentioned that the enzyme maltase was entrapped in calcium alginate beads using 4% sodium alginate- protein mixture and 0.2

Table 1: Effect of concentration of milk on ricin

Concentration of milk in the test sample (%)	Score
Negative Control (NC)	-
Positive Control (PC)	++++
0.125%	++++
0.25%	+++
0.375%	++
0.5%	+
0.625%	-

M calcium chloride solution which exhibited the protein's optimum conditions in its immobilized state (Bibi et al., 2015). In another literature, 4% sodium alginate- xylanase mixture was mixed with 0.4 M calcium chloride solution to yield immobilized beads (Nawaz et al., 2015).

Analysis of the immobilized ricin

Crushed form of immobilized lectins appeared to be slight whitish in color. On incubation with 3% RBC suspension, the Red Blood Cells were found to be deposited on the surfaces of the crushed calcium alginate-lectin slurry. Upon microscopic examination, solid floccules of RBC surrounding the calcium alginate-lectin slurry were observed clearly as in Figure 3. This was due to the interaction of glycoprotein moieties present on the RBC membranes that interact with the lectin molecules causing reversible binding. Likewise, *Solanum melongena* seed lectin could potentially agglutinate the Red Blood Cells of all the four major blood types in humans (Zubčević et al., 2016). The lectin from *Phaseolus coccineus* (ayocote bean) protein extract exhibited its maximum hemagglutination activity of 3618.37 per 131.6 mg of protein (González-Cruz et al., 2022).

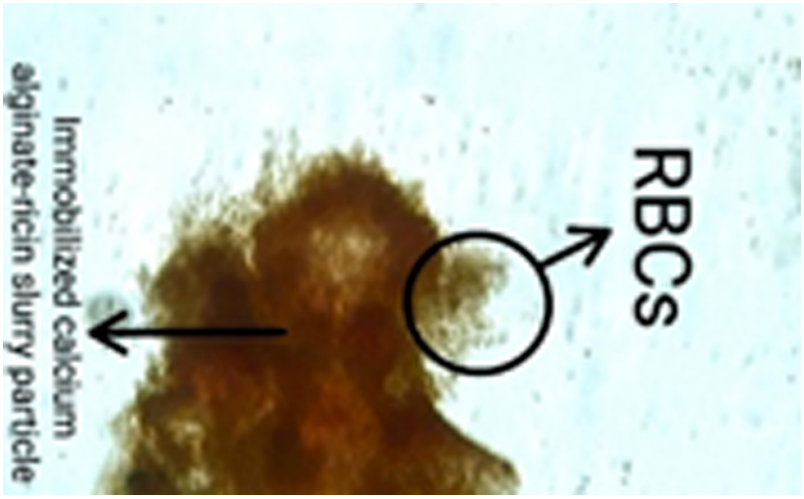

Figure 3. Interaction of RBCs and immobilized ricin

4 Conclusion

The lectin was partially purified from the *Ricinus communis* L. (Castor) seeds and immobilized in the form of calcium alginate beads. Tube Hemagglutination Test (THAT) unraveled that the temperature stability of ricin was found to be 80℃ and when subjected to 95℃, proteins might have denatured leading to a decreased activity (Wood et al., 2018). Milk dependent hemagglutination inhibition test revealed, increasing concentration of milk which contains lactose and galactose in the sample could affect the agglutination of RBCs by ricin due to its inhibitory nature. The crushed slurry form of the calcium alginate-lectin could provide larger surface area for the glycoprotein and glycolipid conjugates to bind and directly interact with each other during the experimental procedures. Hence, immobilized lectin as described above could potentially cause agglutination of the Red Blood Cells. Our research provides a simple strategic methodology for partial characterization and lectin immobilization that finds robust applications in scientific developments such as protein purification, cell immobilization, protoplast immobilization, antimicrobial, immunology and diagnostics (Majee and Biswas, 2013; Nair et al., 2013; Kalidas and Pranav, 2022).

Conflict of interests

The authors declare that they have no conflict of interest.

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References

Al-Saman, M. A., Farfour, S., Tayel, A. A., & Rizk, N. (2015). Bioactivity of lectin from Egyptian *Jatropha curcas* seeds and its potentiality as antifungal agent. Global Advanced Research J. Microbiol, 4, 087–097.

Banger, S., Singh, R., Tripathi, N. K., & Goel, A. K. (2019). One-step Purification and Characterization of Abrin Toxin from *Abrus precatorius* Seeds. Defence Life Science Journal, 4, 231–235.

Bashir, H., Khan, T., Masood, A., & Hamid, R. (2010). Isolation, purification and characterization of a lectin from a local Kashmiri variety of soybean (*Glycine max*). Asian Journal of Biochemistry, 5, 145–153.

Bibi, Z., Qader, S. A. U., & Aman, A. (2015). Calcium alginate matrix increases the stability and recycling capability of immobilized endo-β-1, 4-xylanase from *Geobacillus stearothermophilus* KIBGE-IB29. Extremophiles, 19, 819–819.

Biswas, S., & Kayastha, A. M. (2002). Thermal stability of *Phaseolus vulgaris* leucoagglutinin: a differential scanning calorimetry study. BMB Reports, 35, 472–475.

Blot, L., Passeron, T., & Tulic, M. K. (2022). C-type lectin receptors in skin immunity: Emerging new role for CLEC12B. Advances in Biomedical and Health Sciences, 1, 13–22.

Danielsson, D., & Kronvall, G. (1974). Slide Agglutination Method for the Serological Identification of *Niesseria gonorrhoeae* with Anti-Gonococcal Antibodies Adsorbed to Protein A-Containing Staphylococci. Applied Microbiology, 27, 368–374.

De Mesa, M. D., Mojica, E. R. E., & Merca, F. E. (2004). Purification of lectin from mature seeds of malunggay (*Moringa pterygosperma*). Philippine Journal of Crop Science, 29, 13–24.

González-Cruz, L., Valadez-Vega, C., Juárez-Goiz, J. M. S., Flores-Martínez, N. L., Montañez-Soto, J. L., & Bernardino-Nicanor, A. (2022). Partial Purification and Characterization of the Lectins of Two Varieties of *Phaseolus coccineus* (Ayocote Bean). Agronomy, 12, 716.

Jawade, A. A., Pingle, S. K., Tumane, R. G., & Sharma, A. S. (2016). Isolation and characterization of lectin from the leaves of *Euphorbia tithymaloides* (L.). Tropical Plant Research, 3, 634–641.

Joshi, S. R., Vasantha, K., & Robb, J. S. (2005). An unusual anti-H lectin inhibited by milk from individuals with the Bombay phenotype. Immunohematology, 21, 1–4.

Kalidas, G., & Pranav, R. J. (2022). Biological Synthesis of Metallic Nanoparticles and their Applications. International Journal for Research in Applied Science & Engineering Technology, 10, 420–424.

Lord, J. M., Roberts, L. M., & Robertus, J. D. (1994). Ricin: structure, mode of action, and some current applications. The FASEB Journal, 8, 201–208.

Mahajan, R., Gupta, V. K.,& Sharma, J. (2010). Comparison and suitability of gel matrix for entrapping higher content of enzymes for commercial applications. Indian Journal of Pharmaceutical Sciences, 72, 223–228.

Majee, S. B.,& Biswas, G. R. (2013). Exploring plant lectins in diagnosis, prophylaxis and therapy. Journal of Medicinal Plants Research, 7, 3444–3451.

Nair, S. S., Madembil, N. C., Nair, P., Raman, S.,& Veerabadrappa, S. B. (2013). Comparative analysis of the antibacterial activity of some phytolectins. International Current Pharmaceutical Journal, 2, 18–22.

Nawaz, M. A., Rehman, H. U., Bibi, Z., Aman, A., & Qader, S. A. U. (2015). Continuous degradation of maltose by enzyme entrapment technology using calcium alginate beads as a matrix. Biochemistry and Biophysics Reports, 4, 250–256.

Olsnes, S.,& Pihl, A. (1972). Ricin - a potent inhibitor of protein synthesis. FEBS letters, 20, 327–329.

Purkait, S.,& Koley, S. (2019). Identification and Characterization of Lectins from Leguminosae Plants. International Journal of Health Sciences and Research, 9, 115–121.

Rana, M., Dhamija, H., Prashar, B., & Sharma, S. (2012). *Ricinus communis* L. - A Review. International Journal of PharmTech Research, 4, 1706–1711.

Rao, S. V., Anderson, K. W., & Bachas, L. G. (1998). Oriented immobilization of proteins. Microchimica Acta, 128, 127–143.

Rasooly, R., He, X., & Friedman, M. (2012). Milk Inhibits the Biological Activity of Ricin. Journal of Biological Chemistry, 287, 27924–27929.

Reddy, M. V., Banik, N. L., & Sasikala, P. (2016). Extraction and Purification Of Lectin From Plant Seeds an Empirical Study On *Artocarpus* sp. International Journal of Research in Ayurveda and Pharmacy, 7, 236–240.

Sandvig, K., & Deurs, B. v. (2000). Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. The EMBO Journal, 19, 5943–5950.

Sandvig, K., Olsnes, S., & Pihl, A. (1976). Kinetics of Binding of the Toxic Lectins Abrin and Ricin to Surface Receptors of Human Cells. The Journal Of Biological Chemistry, 251, 3977–3984.

Sharmila, S., Rebecca, L. J., & Saduzzaman, M. (2012). Immobilization of plant protease using calcium alginate beads. Journal of Chemical and Pharmaceutical Research, 4, 4484–4488.

Sharon, N., & Lis, H. (2004). History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology, 14, 53R-62R.

Shinde, S., & Soni, R. (2014). Production and Partial Purification of a-Amylase from Bacterial Strains. International Journal of Genetic Engineering and Biotechnology, 5, 57–62.

Trung, N. N., Tho, N. T., Dung, B. T., Nhung, H. T., & Thang, N. D. (2016). Effects of ricin extracted from seeds of the castor bean (*Ricinus communis*) on cytotoxicity and tumorigenesis of melanoma cells. Biomedical Research and Therapy, 3, 633–644.

Wood, J. P., Richter, W., Smiley, M. A., & Rogers, J. V. (2018). Influence of environmental conditions on the attenuation of ricin toxin on surfaces. PLoS ONE, 13, e0201857.

Youle, R. J., Murray, G. J., & Jr, D. M. (1981). Studies on the galactose-binding site of ricin and the hybrid toxin Man6P-ricin. Cell, 23, 551–559.

Zubčević, N., Suljević, D., Fočak, M., & Rukavina, D. (2016). Effects of plant lectins on human erythrocyte agglutination. Serbian Journal of Experimental and Clinical Research, 17, 207–213.