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Pushpa Choudhary
1. Aquatic Animal Health Management Division, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India
2. Fish Health Management Division, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India

M Makesh
1. Aquatic Animal Health Management Division, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India
2. Finfish Culture Division, ICAR-Central Institute of Brackish water Aquaculture, Chennai, Tamil Nadu, India

Jitendra Kumar
Complement Biology Laboratory, National Centre for Cell Science, S. P. Pune University Campus, Ganeshkhind, Pune, Maharashtra India

Correspondence
Pushpa Choudhary
1. Aquatic Animal Health Management Division, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India
2. Fish Health Management Division, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India

Haemolytic activity of complement component C3 of *Labeo rohita*

Pushpa Choudhary, M Makesh and Jitendra Kumar

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Abstract
Complement component C3 is one of the most abundant complement components found in the plasma/serum of animals and which participates in all three complement activation pathways. In the present study Complement component C3 of rohu, *Labeo rohita* was purified from plasma of rohu by two step column chromatography *viz*, anion and cation exchange chromatography. The purified rohu C3 protein showed the characteristic two chain structure, α and β in reducing SDS-PAGE. The molecular weight of α and β chain was found to be 112 kDa and 77 kDa respectively while in non reducing SDS-PAGE it showed a single band with molecular weight of 189 kDA. The assessment of haemolytic activity of purified rohu C3 was determined by alternative complement pathway and classical complement pathway. Zymosan treated rohu serum showed negligible hemolysis of sheep RBCs while complete hemolysis was observed by addition of purified rohu C3. Dose dependent hemolysis of sheep RBCs was observed in alternative complement pathway as well as in classical complement pathway.

Keywords: Complement C3, *Labeo rohita*, haemolytic assay, zymosan, purification

Introduction

The innate immune system comprises of several components and one of the important component is complement system, which is found in lower as well as higher vertebrates. Complement system consists of more than 35 soluble and membrane-bound proteins and is very efficient in alerting the immune system for the removal of invading pathogens, altered host cells [1-2]. Teleosts, belongs to lower vertebrates but have a well-developed complement system with diversified complement proteins and enhanced efficiency to cope up with the fluctuating environment. Fish complement is active at lower optimal reaction temperature (10-27 °C), can withstand high temperature and has stronger antimicrobial action [3]. Like higher vertebrates such as Mammals fish also have all the three complement activation pathways and fish complement components characterized in other fish species have showed many homologies to their mammalian counterparts [4-5]. These two pathways classical complement pathway and lectin pathway are activated by antibody and pattern recognition molecules (lectins, ficolins) respectively, whereas alternative complement pathway is activated continuously by a tick over mechanism thus providing immune surveillance [1, 6]. Due to presence of effective alternative and classical complement pathway in fish, the fish complement capable of lysing erythrocytes from a number of species and the alternative complement titer in fish is 5-10 times higher than higher animals [7]. From the earlier studies it has been reported that fish complement can lyse foreign cells by formation of membrane attack complex, are able to opsonize foreign organisms for destruction by phagocytes [8].

Complement component C3 is one of the important complement components which participates in all three complement pathways and is most abundant and central complement component present in serum/plasma of fish. C3 is 190 kDa glycoprotein molecule and consists of two disulphide linked α and β polypeptide chain, and in the α-chain there is presence of an intrachain thioester bond between Cys (Cysteine) and Gln (Glutamine) within a GCGEQ sequence motif which is also found conserved in rohu C3 [9]. The proteolytic activation of C3 results in generation of C3a and C3b and these after undergoing gross conformational changes cleaves and expose thioester bond which binds to a hydroxyl group on the target surface by the process of trans acylation [10]. C3 in mammals is encoded by a single gene, while most of the teleosts fish possess multiple form of C3 as a product of different genes [11-19]. However, a

single isotype of C3 has been isolated in Atlantic Cod and Atlantic halibut [20] and wolffish [21] and in carps it is polymorphic in nature [17]. Fish complement having some of the properties such as found in greater diversity, high titer and can get activated in lower temperature, which makes it an effective and efficient immune parameter in fish [18] and these are helpful in expanding innate immune recognition repertoires [16].

Labeo rohita is one of the important candidate species in freshwater aquaculture. The complement component C3 of *L. rohita* is not studied at the protein level although the full length cDNA sequence of C3 and tissue level expression of C3 has been reported recently by Pushpa *et al.* [9]. The present study was undertaken to purify the complement C3 of *L. rohita* and evaluate the structural features of C3 at protein level. The capacity of purified rohu C3 to restore the alternative and classical pathway dependent haemolytic activity of C3- depleted rohu serum was determined for assessing the functional properties.

2. Materials and Methods

2.1 Experimental animal

Healthy rohu (*Labeo rohita*) devoid of any abnormality on the body surface and of average body weight 500-600 g were obtained from Mahad fish farm near Mumbai, India. The acclimatization of fish to the wet laboratory condition was done for one month in 1000 L FRP tanks with continuous aeration and 30% water exchange was done daily. The ambient water temperature was 28±2 °C throughout the rearing period. The fish were fed twice a day with commercial pellet feed.

2.2 Blood collection

The fishes were anaesthetized with clove oil (50 µl/L of water) (Nav Niketan pharmaceutical, India). Blood was collected (pooled from 10 fishes) from the caudal peduncle using 5 ml disposable syringe containing anticoagulant 0.14 M Sodium citrate. Anticoagulant was used at the rate of 0.5 ml per 5 ml of blood. Centrifugation of anticoagulated blood was carried out at 3000 x g for 10 min and supernatant was again centrifuged at 8500 x g for 10 min to separate plasma. The plasma was separated and stored at -70 °C until further use. For serum separation, another ten fishes were used for collection of blood. Whole blood without the use of anticoagulant was drawn from the caudal peduncle using 5 ml syringe with a 24 gauge needle. The blood was allowed to clot and kept at 4 °C for 4 h. The blood samples were centrifuged at 2000 x g for 10 min and the serum was separated and stored at -70 °C until further use.

2.3 Purification of C3 from rohu plasma

Complement C3 protein is prone to fast degradation. Several precautionary measures such as additions of phenylmethane sulfonyl fluoride (PMSF), Benzamidine and Ethylenediaminetetraacetic acid (EDTA) were used to prevent proteolytic degradation and to ensure that C3 remains intact and functionally active. Purification of complement component C3 protein was done by following the method described by Nakao *et al*., [17] with partial modifications. C3 purification was done using two chromatographic steps ie., anion and cation exchange chromatography. The rohu plasma samples and eluted fractions were always kept on ice while performing chromatography. Polyethylene glycol (PEG) precipitation of 10 ml of plasma sample was done by stirring with 4% PEG (MW 8000) at 4 ºC for 30 min in the presence of 20 mM EDTA, 10 mM benzamidine hydrochloride and 1 mM PMSF, and centrifuged (15,000 x *g*, for 20 min). The supernatant obtained after centrifugation was subjected to 16% PEG precipitation by stirring at 4 ºC for 30 min, and then the same solution was centrifuged at 15,000 x *g*, for 20 min and obtained pellet was resuspended in 10 mM phosphate buffer, pH 7.5. 2 ml samples were applied to an anion exchange chromatography column (Mono Q HR 5/5, Pharmacia Biotech, Sweden) which was previously equilibrated with 10 mM phosphate buffer, pH 7.5 and the bound proteins were eluted with a linear gradient of 0-500 mM NaCl. Fractions (2 ml) were collected at a flow rate of 2 ml min^{-1} and the protein concentration was measured by a BCA protein measurement kit (New England Bio-Labs Inc., Beverly, MA, USA). The fractions were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gel under reducing as well as non-reducing conditions and C3 was visualized by coomassie brilliant blue staining. The C3-rich pools, were concentrated using phosphate buffered saline (pH 7.2) at 4 ºC ten times and applied to the cation exchange column (Mono S, Pharmacia, Sweden) which was equilibrated in 10 mM sodium phosphate buffer at pH 6. Elution of the bound proteins was carried with a linear gradient of 0-500 mM NaCl and run with a flow rate of 2 ml min^{-1}.

2.3.1 SDS-PAGE

SDS-PAGE of proteins was carried out using the Mini-PROTEAN II system (Bio-Rad, USA) following the method described by Laemmli [22]. Electrophoresis of samples were done under reducing conditions where samples were denatured with a sample buffer containing 0.5% 2-mercaptoethanol, 20% glycerol and 2% SDS in 0.5 M Tris, pH 6.8, for 5 min at 100 ºC. For samples electrophoresed under non-reducing conditions the same sample buffer, omitting the 2-mercaptoethanol, was used. The electrophoresis was carried out in 4.5% stacking and 7.5% resolving polyacrylamide gel in 25 mM Tris–glycine buffer pH 8.8, containing 0.1% SDS. The staining of gels was done in 0.1% Coomassie Brilliant Blue R-250. Purified human C3 (Sigma Aldrich, USA) was also electrophoresed along with elutes to identify the C3 fractions of rohu.

2.4 Preparation of C3-depleted serum

Zymosan (Sigma Aldrich, USA) was used for preparing C3-depleted rohu serum. Briefly 100 mg of zymosan was dissolved in gelatin veronal buffer (GVB) and washed in the same buffer by centrifuging at 1200 x g for 5 min. The pellet obtained after centrifugation was resuspended in 120 µl of rohu serum and was diluted at the ratio of 1:5 in GVB then incubated for 30 min at 28 °C. After incubation period is over the serum sample was centrifuged and the supernatant collected was used for haemolytic assay. The amount of zymosan used was equivalent to approximately 0.8 g ml^{-1} serum.

2.4.1 Preparation of rohu antiserum against sheep red blood cells (ShRBCs)

Rohu antiserum against sheep red blood cells was prepared by following the method described by Boshra *et al*., [23]. Briefly, fresh sheep RBCs were collected and counted by haemocytometer. 0.2 ml of ShRBCs ($5x10^8$) mixed with equal volume of Freund's complete adjuvant (FCA) (Bangalore

Genei, India) was injected to fish weighing 40-50 g after one month, the booster dose containing $1x10^8$ cells in Freund's incomplete adjuvant (FIA) (Bangalore Genei, India) and after two months blood from immunised fish was drawn from the caudal peduncle using a 26-gauge needle. Antiserum was collected as described by Sunyer *et al*., [12] and stored at -70 °C until further use.

2.4.2 Sensitization of ShRBCs

The sensitization of ShRBCs was done as follows. $1x10^9$ cells of ShRBCs taken after counting by haemocytometer and incubated in 2.5 ml of 3% rohu ShRBc antiserum in PBS with 10 mM EDTA (pH 7.5) for 1 h. The cells were washed twice with PBS containing 10 mM EDTA, followed by two washes with PBS. The sensitized cells were then resuspended at a concentration of $5x10^8$ cells/ml prior to further use.

2.4.3 Haemolytic assay

Assessment of the haemolytic activities of purified rohu C3 was done by evaluating their ability to reconstitute the alternative and classical pathway-mediated haemolytic activity of zymosan-treated rohu serum by following the method described by Boshra *et al*., [23]. Briefly, 25 µl of either sensitized or nonsensitized ShRBCs ($1.5x10^6$ cells) was incubated in 125 µl zymosan- treated rohu serum which was dissolved earlier in 10 mM Veronal-buffered saline with 5 mM Ca^{2+}/Mg^{2+} (pH 7.3). Different concentrations (0-200 µg/ml C3) were added to the zymosan - treated rohu serum containing ShRBCs. Optical density was measured in spectrophotometer at 405 nm. Calculation of haemolytic activities was done as percentage of the degree of haemolysis of ShRBCs incubated with control rohu serum in 5 mM Ca^{2+}/Mg^{2+} (pH 7.3).

3. Results

3.1 Purification and characterisation of rohu C3

In the present study, the diluted antiprotease treated and polyethylene glycol precipitated plasma was loaded through an anion exchange column and the elution of bound proteins were done using 0-500 mM linear NaCl gradient (Fig. 1A). The analysis of eluted fractions from anion exchange column were determined by reducing and non-reducing sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B and Fig. 1C) which revealed several bands in addition to the C3 fractions. In the fig 1B and 1C there are multiple bands are seen in all the alternate elutes, as this was first step for purification of rohu plasma containing complement C3 protein and the last lane is positive control, human C3. Non reducing SDS-PAGE of eluted fractions obtained after cation exchange chromatography purification showed a single band with molecular weight of 189 kDA (Fig. 2B) while reducing SDS-PAGE analysis showed two bands (α and β chains) with molecular weights of 112 kDa and 77 kDa, respectively (Fig 2A). 10 ml of rohu plasma was used for complement C3 purification and a total of 500 µl of purified complement C3 with a concentration of 3.1 mg ml^{-1} was obtained. Purification of complement C3 directly from plasma by chromatography usually results in the isolation of C3 with poor functional activity.

3.2 Hemolytic assay

To assess the functional properties of the purified rohu C3 molecule, alternative and classical pathway dependent haemolytic activity of C3 - depleted rohu serum was evaluated by its capacity to restore the haemolysis of foreign erythrocytes. Complement C3 depleted rohu serum was prepared by treatment of serum with zymosan and purified rohu C3 protein was added concurrently to the zymosan-treated serum. Determination of alternative haemolytic pathway activity was carried out by measuring percent-haemolysis of non-sensitized ShRBCs (Fig. 3A) and classical pathway activity was estimated by measuring percent-haemolysis of sensitized ShRBCs (Fig. 3B). The haemolytic activity of the zymosan-treated rohu serum was almost negligible by alternative and classical pathway pathways, and purified C3 was required to restore the haemolytic activity for both alternative pathway activity and classical pathway activity. The restoration of haemolytic activity as shown in fig 3 A-B, was obtained in a dose dependent manner with addition of increasing amount of purified rohu C3 protein. However, the complete haemolytic activity was obtained when sensitised ShRBCs were used.

4. Discussion

Complement system is one of the important humoral component present in serum/plasma of fish and is able to interacts critically with some other mechanisms of innate immunity as well as with adaptive immunity to enhance host defence. Complement component C3 is the common component which actively participates in all the three complement activation pathways *viz*., classical complement pathway, alternative complement pathway and lectin pathway. Complement C3 has been studied and characterized in detail in all major vertebrates [24] as well as in some of the invertebrate species such as ascidians [25] and the sea-urchin [26].

In the present study, complement C3 of rohu was purified by using two step column chromatography. Purification of complement C3 directly from plasma by chromatography methods usually results in the isolation of C3 with poor functional activity. Tack and Prahl [27] developed a method for the purification of human C3 by fractionation with polyethylene glycol (PEG) of fresh plasma in the presence of protease inhibitors such as Benzamidine hydrochloride followed by sequential depletion of plasminogen by affinity adsorbents, making it possible to keep the functional activity intact. Therefore, the same method was followed in the purification of complement C3 from rohu plasma. The isolated rohu C3 had the characteristic features of complement C3 protein such as molecular weight that is comparable with other teleost fish as well as higher vertebrates. Reduction of the purified C3 protein with β-mercaptoethanol yielded a characteristic two chain structure of α and β polypeptide chains of 112 and 77 kDa. Complement C3 purification from number of teleosts [11-12, 17, 20] as well as higher vertebrates such as human [28] has shown characteristic two chain structure of C3.

Generally complement - mediated haemolysis of foreign erythrocytes occurs when the lytic pathway is activated. As evident from earlier studies [7], in contrast to human complements, complement systems of different species of fish are able to lyse the RBCs of various animals such as sheep, goat, dogs and humans efficiently through alternative complement pathway. The classical pathway is activated when sensitized foreign erythrocytes are used. The purified rohu C3 protein involvement in haemolytic activity of C3 depleted rohu serum was assessed by both alternative complement pathway activity and classical complement

pathway activity. As evident from fig 3 A-B, zymosan treatment of rohu serum resulted in elimination of haemolytic activity by both alternative pathway activity and classical pathway activity and addition of purified C3 to the C3 depleted rohu serum restored haemolytic activity through both alternative and classical pathways. Our result is in agreement with one of the earlier study by Nakao *et al.* [18] where they reported that purified complement C3 of common carp is capable of restoring the haemolytic activity by classical pathway. In another study where the depletion of complement component C3 in fish serum was accomplished by treatment with methylamine and the restoration of haemolytic activity in C3 depleted serum was obtained by addition of purified C3-1 complement protein [23].

5. Conclusion

The present study reports the purification of rohu complement component C3 and hemolytic assay by alternative complement pathway and classical complement pathway using purified C3 in *L. rohita* for the first time. The results obtained and presented in this study contributes to the basic knowledge of the complement system and innate immunity of rohu.

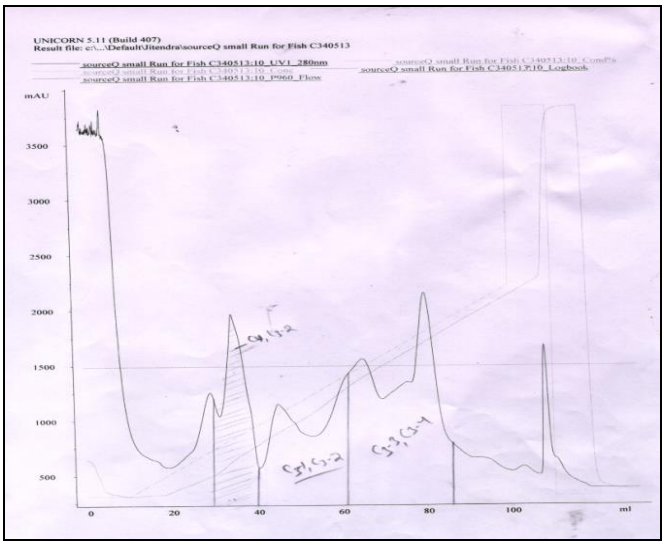

Fig 1-A: Elution profile of C3 protein after anion exchange chromatography.

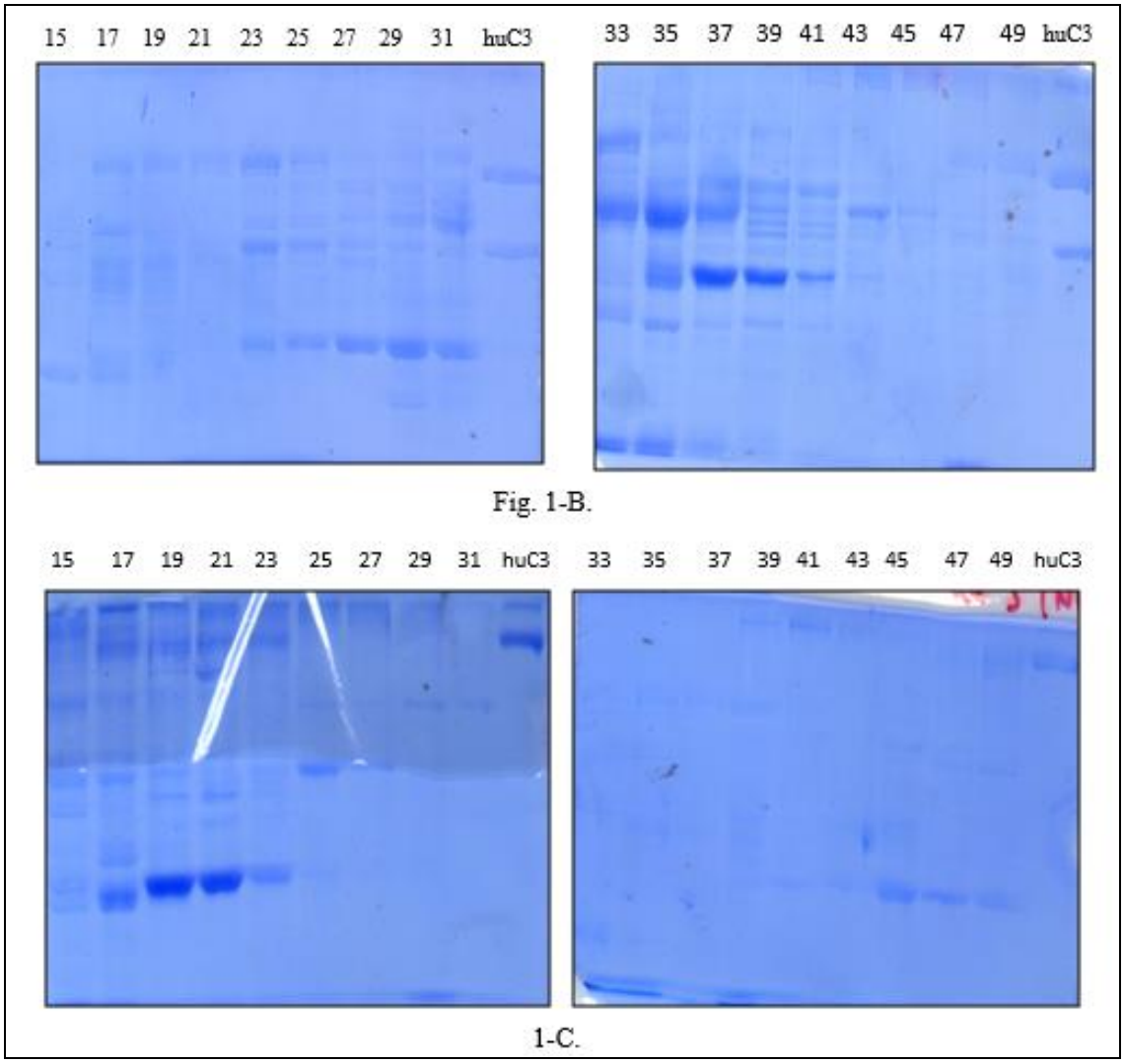

Fig 1: B. and C. SDS-PAGE of C3-containing alternate fractions after anion exchange chromatography were determined under reducing and non-reducing conditions with 7.5% SDS-PAGE respectively.

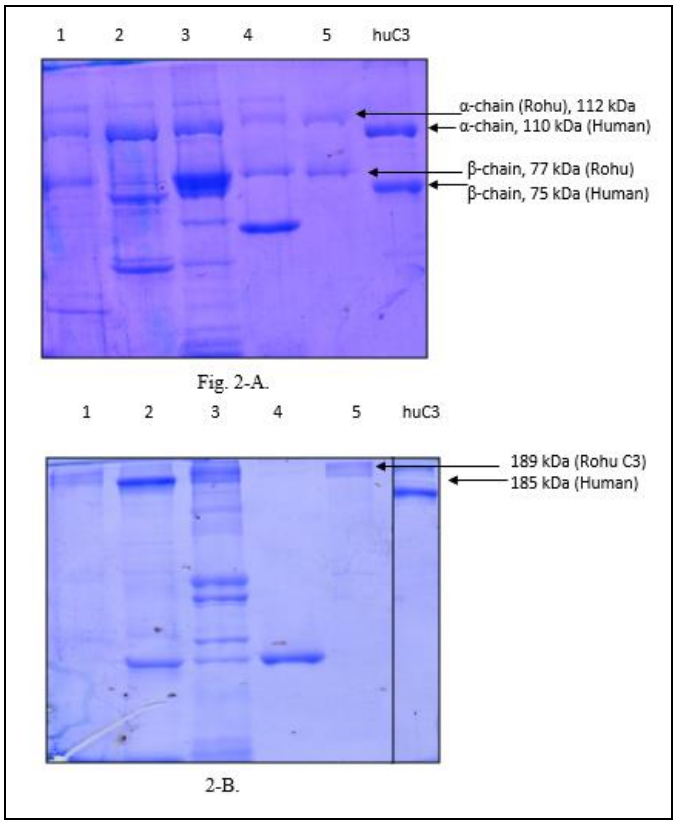

Fig 2: A-B. C3-containing concentrated protein obtained after 2nd chromatography step (Cation exchange) were determined under reducing and non-reducing conditions with 7.5% SDS-PAGE respectively. The purified C3 is shown in lane 5 (α-chain, 112 kDa, β-chain, 77 kDa) corresponding to human C3 in reducing gel. In non-reducing gel, 189 kDa C3 is shown in lane 5.

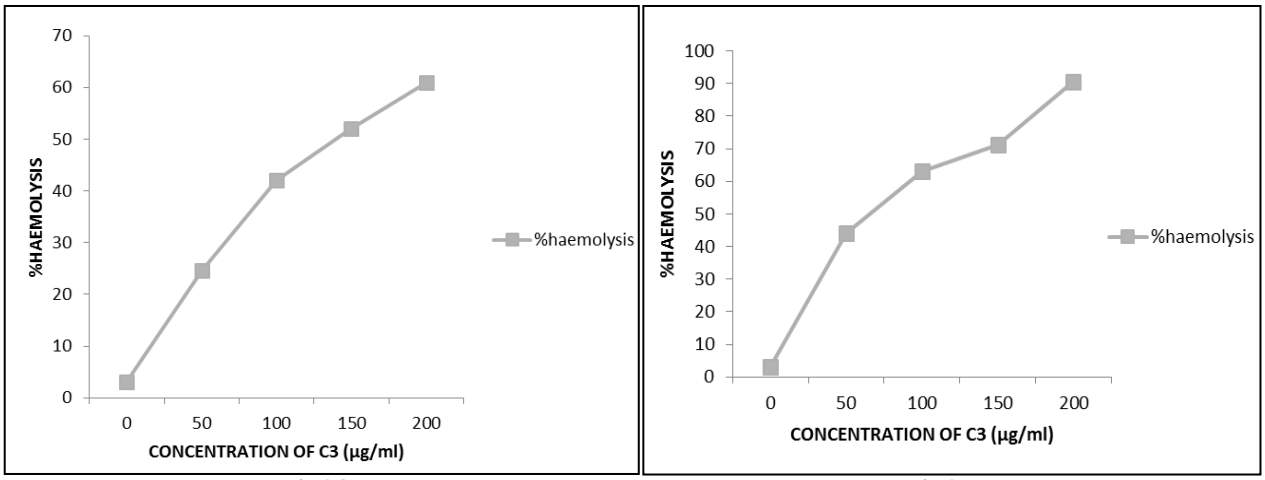

Fig 3A **Fig 3B**

Fig 3: Lysis of (A.) un-sensitized and (B.) sensitized sheep RBCs with C3-depleted rohu serum by addition of purified rohu C3. Depletion of C3 in Rohu serum by zymosan treatment, and its haemolytic activity through alternative and classical pathway was restored by adding various amounts of purified rohu C3.

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