Check for updates

[*This is a protocol for inclusion in CPPS volume 101. There are 9 figures and 0 tables included. No PMC file is required.]

Author Manuscript

A rapid and facile purification method for glycan-binding proteins and glycoproteins

Christina J. Welch[1], Priyanka D. Kadav[1], Jared L. Edwards[1], Jessica Krycia[1], Melanie L. Talaga[1#], Purnima Bandyopadhyay[1], and Tarun K. Dam[1,2*]

From the [1]Laboratory of Mechanistic Glycobiology, Department of Chemistry; [2]Health Research Institute, Michigan Technological University, Houghton, MI 49931

[#]Present address: College of Saint Scholastica, Duluth, MN 55811

*To whom correspondence should be addressed: Tarun K. Dam: [1]Department of Chemistry, Michigan Technological University, Houghton, MI 49931; tkdam@mtu.edu; Tel. (906) 487-2940; Fax: (906) 487-2061.

Running head: Capture and release

Keywords: Galectin-3, lectin, glycoprotein purification, protein purification, affinity chromatography, non-covalent cross-linking, agglutinin, proteomics, glycoproteomics, method development

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/cpps.113.

This article is protected by copyright. All rights reserved.

ABSTRACT:

Glycosylated proteins, namely glycoproteins and proteoglycans (collectively called glycoconjugates), are indispensable in a variety of biological processes. Functions of many glycoconjugates are regulated by their interactions with another group of proteins known as lectins. In order to understand the biological functions of lectins and their glycosylated binding partners, one must obtain these proteins in pure form. The conventional protein purification methods often require longer time, elaborate infrastructure, costly reagents, and larger sample volume. To minimize some of these problems, we recently developed and validated a new method termed Capture and Release (CaRe). This method is time-saving, precise, inexpensive, and it needs relatively small sample volume. In this approach, targets (lectins and glycoproteins) are captured in solution by multivalent ligands called target capturing agents (TCAs). The captured targets are then released and separated from their TCAs to obtain purified targets. CaRe could play important roles in discovering new lectins and glycoconjugates.

Author Manuscript

This article is protected by copyright. All rights reserved.

Basic Protocol 4: Identification of multivalent inhibitors as TCAs by hemagglutination inhibition assays

Basic Protocol 5: Testing the capturing abilities of TCAs by precipitation/turbidity assays

Basic Protocol 6: Capturing of targets (lectins and GPs) in the crude extracts by TCAs and separation of the target-TCA complex from other components of the crude extracts

Basic Protocol 7: Releasing the captured targets (lectins and GPs) by dissolving the complex

Basic Protocol 8: Separation of the targets (lectins and glycoproteins) from their respective TCAs

Basic Protocol 9: Verification of the purity of the isolated targets (lectins or GPs)

INTRODUCTION:

Glycoproteins (GPs) and proteoglycans (PGs) constitute a major domain of the protein family (Stanley et al., 2017; Brockhausen and Stanley, 2017; Lindahl et al., 2017). They are involved in a variety of cellular and extracellular functions (Varki, 2017). The biological activities of GPs and PGs are often initiated when their covalently linked glycan chains interact with another group of proteins called glycan binding proteins (GBPs) (Taylor et al. 2017). GBPs are broadly divided into two subgroups: lectins and glycosaminoglycan-binding proteins (GAGBPs) (Taylor et al. 2017; Esko, et al., 2017). While lectins bind to the glycan chains of GPs, GAGBPs interact with the constituent glycosaminoglycan chains of PGs. To understand the biological functions mediated by GP-lectin and PG-GAGBP interactions, these proteins must be available in purified forms. Current approaches to purify these macromolecules include affinity column chromatography (Arora et al., 2017), size exclusion chromatography (Burgess, 2018) and ion exchange chromatography (Cummins et al., 2017). Such purification methods often require elaborate infrastructure, larger volume of starting crude samples, longer operational time and covalent modifications of the reagents. These methods are also multi-step and expensive. Other drawbacks associated with these approaches include nonspecific binding and the risk of losing the target proteins.

Author Manuscript

This article is protected by copyright. All rights reserved.

Immunoprecipitation (IP) (Speth et al., 2014; Lin and Lai, 2017; Kaboord, and Perr, 2008) is another technique that can identify/purify proteins form limited sample volumes. However, the use of IP is very limited for the above-mentioned proteins. In addition, IP requires costly antibodies and a substantial amount of prior information regarding the target proteins. Harsh dissociation (elution) conditions and non-specific binding in IP can also produce ambiguous results.

In order to overcome some of the problems described above, we formulated and validated a new method named "Capture and Release" (CaRe) for lectin and GP purification (Welch, et al. 2020). In this approach (Figure 1), target-capturing agents (TCAs) capture targets (lectins or GP) in solution phase through multivalent glycan-protein interactions and form insoluble complexes. The captured targets are released from the complexes by competitive monovalent ligands. The released targets are then separated from the TCAs by membrane filtration or gel filtration. CaRe is a relatively rapid and simple method that does not require any affinity matrices, specialized detectors, customized apparatus, controlled environments or covalent modification of the reagents. This method can purify lectins and GPs from a few milliliters of crude extracts by using nominal infrastructural support and limited space. Thus, CaRe has the potential to expedite the discovery of lectins, glycoconjugates or any other targets that engage in multivalent binding with their specific ligands.

[*Place figure 1 near here.]

STRATEGIC PLANNING

Identifying and collecting reliable sources (plant and animal) of lectins and glycoproteins (targets): The first major step of this protocol is to identify the sources of the target lectins and glycoproteins (targets). If the targets are known, their sources are collected for crude preparation by aqueous extraction. For discovering new targets, sources of interest are identified based on the scientific questions, objectives and priorities.

Creating a panel of monovalent and multivalent ligands: In this protocol**,** suitable multivalent ligands act as target capturing agents (TCAs), whereas the specific monovalent ligands are used as

Author Manuscript

This article is protected by copyright. All rights reserved.

releasing agents. Therefore, the success of this protocol critically depends on the appropriate selection of the ligands. For known targets, a panel of potentially specific and relevant ligands is created. For unknown targets, the panel contains a wide variety of ligands with different structural attributes that could be recognized by lectins of different specificity.

Having access to fresh red blood cells (RBCs) of rabbits: For CaRe, fresh rabbit RBCs are essential for determining lectin activity as well as for selecting the TCAs and the releasing agents. Therefore, a steady supply of RBCs is required.

Selecting the proper TCAs: An important factor to consider during the selection of TCAs is their molecular weight. The molecular weights of the TCAs should differ from their targets (at least by 10 kDa). This is essential for proper separation of the targets from their TCAs.

Knowledge of glycosylated proteins and their receptors: Importance of a clear concept about glycosylated proteins and their receptors cannot be overstated, especially when the targets are unknown. It helps solving any unanticipated problems one might encounter when performing the protocol.

Basic Protocol 1

Preparation of Crude Extracts containing the target proteins from Soybean flour

Proteins, native or recombinant, are generally purified from crude preparations. In the present study, lectins and GPs were purified from crude extracts and cell lysates. These extracts were prepared from plant tissues (seeds, bulbs and corms), *E. coli* cells and chicken eggs. The main objectives of crude preparation are to solubilize the target proteins in aqueous buffer, make a clear solution by discarding the cell and tissue debris, and enrich the target proteins, whenever possible. Another important aspect of crude preparation is to make sure that the target proteins are indeed present in the extracts and they are active. This is achieved by checking the crude for the signature activities of the target proteins (for example, the signature activity of lectins is hemagglutination. See Basic Protocol 3). The purpose of this procedure is to produce workable crude extracts that can

Author Manuscript

This article is protected by copyright. All rights reserved.

be used for the capture and isolation of the target proteins. Image of a representative crude extract is shown in Figure 2.

The lectin soybean agglutinin (SBA) is found in soybean. Soybean flour prepared from soybean is commercially available. SBA is purified by affinity chromatography from commercially available flour in a laboratory set-up. The first step of purification is the preparation of crude extract from the flour. Properly prepared crude solution is required for CaRe.

[*Place figure 2 near here.]

Materials:

Soybean flour from Millipore Sigma (Catalog #S9633)

Luria Bertani media (Fisher Scientific, Millipore Sigma)

Hexane (Millipore Sigma)

20 mM Phosphate buffered saline (PBS, see recipe in **Reagents and Solutions**)

Ammonium Sulfate from Millipore Sigma (Catalog #A3920)

Dialysis Sacs from Spectrum labs (Catalog # 132650)

Dialysis Sac Clips

Beakers of various sizes

Erlenmeyer flasks

Graduate Cylinders of various sizes

Spatula

Author Manuscript

This article is protected by copyright. All rights reserved.

50 mL centrifuge tubes

Magnetic stir bars

Magnetic stir plate

Standard analytical balance

Sonicator (Fisher Scientific)

Blender (Oster Duralast Classic)

Centrifuge (Thermo Scientific Sorvall Legend X1R)

1. Tare a 1 L beaker on a standard analytical balance.
2. Weigh 100 g of Soybean flour into the beaker.
3. Remove the beaker from the analytical balance and place a magnetic stir bar inside of it. Set aside on the lab bench.
4. Measure 600 ml of hexane into a graduated cylinder
5. Pour the hexane into the beaker containing soybean flour and let the mixture stir on a stir plate at room temperature for 3 hours.
6. After 3 hours, remove the mixture from the stir plate and place it on the lab bench.
7. Let the mixture settle until you have a hexane layer and a soybean flour layer.
8. Gently decant the hexane into a separate waste container.
9. Place the beaker containing the soybean flour layer into the fume hood for air-drying.
    a. *Remove the stir bar before placing it into the fume hood.*
    b. *Keep the beaker in the fume hood up to 72 hours to completely dry the flour.*
10. When the flour is dry, disturb it by using a scoopula until it is no longer uniform and set the beaker aside.
11. Measure 500 ml of PBS (pH 7.4) into a graduated cylinder.
12. Pour the PBS into the dry flour and add a stir bar.
13. Let the mixture stir on a stir plate overnight at 4°C.
14. Remove the mixture from 4°C and decant it into 50 mL centrifuge tubes.
15. Centrifuge the tubes on 1957 × *g* for 30 minutes at 4°C.

Author Manuscript

This article is protected by copyright. All rights reserved.

16. Remove the tubes from the centrifuge and pool the supernatant together. Discard the precipitate.
17. Centrifuge the supernatant again on 9469 × *g* for 30 minutes at 4°C.
18. Pool the supernatant together in a separate beaker and temporarily store at 4°C.
19. Weigh out 164 g/liter (30%) of solid ammonium sulfate into a beaker.
20. Pour the supernatant into the ammonium sulfate and add a stir bar into the mixture.
21. Stir the mixture on a stir plate for 3 hours at 4°C.
22. After stirring, centrifuge the mixture on 9469 × *g* for 30 minutes at 4^0 C.
23. Pool the supernatant together and temporarily store it at 4^0 C.
24. Weigh out 516 g/liter (80%) of solid ammonium sulfate into a beaker.
25. Pour the supernatant into the ammonium sulfate and add a stir bar into the mixture.
26. Stir the mixture on a stir plate overnight at 4^0 C.
27. Centrifuge the mixture on 9469 × *g* for 30 minutes at 4^0 C.
28. Discard the supernatant.
29. Dissolve the precipitate in PBS and dialyze the solution until the ammonium sulfate concentration is less than 0.01 nM (determined from the approximate initial concentration of ammonium sulfate in the dissolved precipitate multiplied by the volume of the dissolved precipitate and then the product is divided by the total volume of buffer the precipitate was dialyzed against. Depending on the volume of the dissolved precipitates and the amount of ammonium sulfate used, it takes 2 to 4 days and 8 to 12 liters of PBS to complete the dialysis).
30. Use the dialyzed sample for CaRe.

Alternate Protocol 1. Preparation of crude extracts from Jack bean meal

Jack bean is the source of the lectin Concanavalin A (ConA). ConA is purified by affinity chromatography from commercially available Jack bean or Jack bean meal. The first step of purification is the preparation of crude extract by using the beans or the meal. ConA was purified by CaRe from the crude extract of Jack bean.

Additional Materials

Jack Bean from Millipore Sigma (Catalog #J0250)

This article is protected by copyright. All rights reserved.

Author Manuscript

100 mM HEPES buffer (HEPES, see recipe in **Reagents and Solutions**)

100 mM Sodium acetate (with ions) buffer (see recipe in **Reagents and Solutions**)

Sodium acetate buffer (see recipe in **Reagents and Solutions**)

1. Tare a 600 ml beaker on a standard analytical balance.
2. Weigh 250 g of jack beans into the beaker.
3. Remove the beaker from the balance and set aside on the lab bench.
4. Measure 300 ml of HEPES buffer (pH 7.2) into a graduated cylinder.
5. Pour the HEPES buffer into the beaker and cover with aluminum foil.
6. Let the jack beans soak in the HEPES buffer overnight at 4°C.
7. Remove the beaker from 4°C and set it on the lab bench.
8. Gently decant the HEPES buffer from the beans.
9. Remove the skin from the beans and place the beans in a separate 1 L beaker.
10. Grind the beans in a blender and slowly add 100 ml of HEPES buffer five times.
    a. *Continue to add HEPES buffer to the mixture until it has a moderate consistency.*
11. Pour the homogenized mixture into a beaker and add a stir bar.
12. Stir the mixture on a stir plate overnight at 4°C.
13. Remove the beaker from 4°C and decant the mixture into 50 mL centrifuge tubes.
14. Centrifuge the tubes on 1957 × *g* for 30 minutes at 4°C.
15. Remove the tubes from the centrifuge and pool the supernatant together. Discard the precipitate.
16. Centrifuge the supernatant again on 9469 × *g* for 30 minutes at 4°C.
17. Pool the supernatant together in a separate beaker and temporarily store at 4°C.
18. Weigh out 164 g/liter (30%) of solid ammonium sulfate into a beaker.
19. Pour the supernatant into the ammonium sulfate and add a stir bar into the mixture.
20. Stir the mixture on a stir plate overnight at 4°C.
21. After stirring, centrifuge the mixture on 9469 × *g* for 30 minutes at 4^0 C.
22. Pool the supernatant together and temporarily store it at 4^0 C.
23. Weigh out 516 g/liter (80%) of solid ammonium sulfate into a beaker.
24. Pour the supernatant into the ammonium sulfate and add a stir bar into the mixture.

Author Manuscript

This article is protected by copyright. All rights reserved.

25. Stir the mixture on a stir plate overnight at 4°C.
26. Centrifuge the mixture on 9469 × *g* for 30 minutes at 4^0 C.
27. Discard the supernatant and dissolve the precipitate in 100 ml of HEPES buffer.
28. Dialyze half of the mixture against HEPES buffer.
29. Dialyze the other half of the mixture against sodium acetate buffer (pH 5.2).
30. Continue the dialysis until the ammonium sulfate concentration is less than 0.01 nM.
31. Use both of the dialyzed samples for CaRe.

Alternate Protocol 2. Preparation of crude extracts from the corms of Colocasia esculenta, Xanthosoma sagittifolium, and from the bulbs of Allium sativum (sources of C. esculenta lectin, X. sagittifolium lectin, Allium sativum agglutinin, ASA)

Corms of *Colocasia esculenta*, *Xanthosoma sagittifolium*, and bulbs of *Allium sativum* contain *C. esculenta* lectin, *X. sagittifolium* lectin, and *Allium sativum* agglutinin, ASA, respectively. These corms and bulbs, obtained from the local market, were used to prepare crude extracts. Lectins from respective extracts were purified by CaRe.

Additional Materials

Corms of *Colocasia esculenta* (From local vendors)

Corms of *Colocasia esculenta* (From local vendors)

Corms of *Xanthosoma sagittifolium* (From local vendors)

Bulbs of *Allium sativum* (From local vendors)

100 mM HEPES buffer (HEPES, see recipe in **Reagents and Solutions**)

100 mM Sodium acetate (with ions) buffer (see recipe in **Reagents and Solutions**)

Sodium acetate buffer (see recipe in **Reagents and Solutions**)

Author Manuscript

This article is protected by copyright. All rights reserved.

1. Grind the corms of *C. esculenta, Xanthosoma sagittifolium,* or *Allium sativum* and slowly add 500 ml of the perspective buffer.
    a. *PBS is the most suitable buffer when the CaRe is done at the physiological pH (ph ~7.4). However, if a lectin requires any metal ions for activity, HEPES should be used. If CaRe is done at acidic pH, then sodium acetate buffer should be used.*
    b. *Continue to add the buffer until the mixture has a moderate consistency.*
2. Pour the homogenized mixture into a beaker and add a stir bar.
3. Stir the mixture on a stir plate overnight at 4°C.
4. Remove the beaker from 4°C and decant the mixture into 50 mL centrifuge tubes.
5. Centrifuge the tubes on 1957 × *g* for 30 minutes at 4°C.
6. Remove the tubes from the centrifuge and pool the supernatant together. Discard the precipitate.
7. Centrifuge the supernatant again on 9469 × *g* for 30 minutes at 4°C.
8. Remove the tubes from the centrifuge and pool the supernatant together. Discard the precipitate.
    a. *Repeat steps 7 and 8 until the solution is transparent or translucent.*
9. Use this sample for CaRe.

Alternate Protocol 3. Preparation of E. coli cell lysates containing human Galectin-3 (Gal-3)

Recombinant human Gal-3 was expressed and produced by *E. coli*. The bacterial cells containing expressed Gal-3 were disrupted by sonication in order to release the Gal-3 along with other cellular components of *E. coli*. The cell extract, thus prepared, was used as the source of Gal-3, which was subsequently purified by CaRe.

Additional Materials

E. coli (BL21 DE3) transformed with a plasmid (commercially obtained) expressing human Gal-3

Author Manuscript

This article is protected by copyright. All rights reserved.

Sonication buffer (22 mM Tris-HCl (pH 7.5) with 5 mM EDTA, protease cocktail inhibitor added immediately prior to use)

1. Centrifuge *E. coli* (BL21 DE3) transformed with a plasmid expressing human Gal-3 at 1957 x g for 30 minutes at 4^{o}C in order to obtain the cell pellets from 1 to 3 liters of culture.
2. Sonicate the cell pellets in 20 to 60 ml of sonication buffer [22 mM Tris-HCl (pH 7.5) with 5 mM EDTA] containing protease cocktail inhibitor (10μL/ml of sonication buffer). The sonication should be done on ice and at 50% Amplitude for 8 cycles of 30 seconds each with 1-minute interval in between two cycles.
3. Centrifuge the sonicated cell lysate at 1957 x g for 30 minutes at 4°C. Collect the supernatant.
4. Centrifuge the supernatant again at 9469 × g for 30 minutes at 4°C. Collect the supernatant.
5. Use this supernatant to purify Galectin-3 by CaRe.

Alternate Protocol 4. Preparation of crude extracts from chicken egg whites (source of ovalbumin)

Chicken egg whites contain the glycoprotein ovalbumin. Therefore, commercially available raw chicken eggs are used as the source of ovalbumin. After separating the yolks, the viscous white part is clarified with buffer to produce the crude extract. Ovalbumin is purified by CaRe from this extract.

Additional Materials

Eggs (from local vendors)

100 mM HEPES buffer (HEPES, see recipe in **Reagents and Solutions**)

1. Place a beaker on the lab bench.

This article is protected by copyright. All rights reserved.

Author Manuscript

2. Carefully separate the chicken egg yolks from egg whites while placing the egg whites into the beaker. [*Copy Editor: Please query the authors to determine how many egg whites should be used in this assay.]
3. Decant the egg whites into a blender and add 50 ml of HEPES buffer.
4. Blend the mixture for 30 seconds three times (90 seconds total).
5. Decant the mixture into a beaker and add a stir bar.
6. Stir the mixture on a stir plate for 2 hours at 4°C.
7. After 2 hours, decant the mixture into 50 ml centrifuge tubes.
8. Spin the mixture on 1957 × *g* for 30 minutes at 4°C.
    a. *Observing a small amount of precipitate is normal*
9. Pool the supernatant together and spin the mixture again on 9469 × *g* for 30 minutes at 4°C.
10. Pool the supernatant together. Discard the precipitate.
11. Use the supernatant for CaRe.

Basic Protocol 2

Preparation of 2% v/v Red Blood Cell (RBC) Suspension

The ability to agglutinate mammalian red blood cells (hemagglutination) is a characteristic feature of lectins. Therefore, hemagglutination tests are performed to determine the presence of lectins in any biological samples. This test is also done to assess the level of activity of lectins. RBCs from different mammalian species could be used for hemagglutination tests. However, rabbit RBCs are the most commonly used because a wide variety of lectins agglutinate them. Rabbit blood is drawn in the presence of heparin, thoroughly washed and then fixed volume (most commonly 2% v/v) of RBCs is suspended in working buffer. This 2% (v/v) RBC solution was used for all the hemagglutination tests in the present study. Basic Protocol 2 describes how 2% (v/v) rabbit RBC solution is made. Image of 2% (v/v) rabbit RBC solution is shown in Figure 3.

[*Place figure 3 near here.]

This article is protected by copyright. All rights reserved.

Author Manuscript

Materials:

Unwashed rabbit blood from rabbits maintained at Michigan Tech Animal Care Facility

Glass Pipette

15 mL Centrifuge Tubes

20 mM PBS buffer (see recipe in **Reagents and Solutions**)

Micropipette

Micropipette tips

Centrifuge

2. (a) Washing the Red Blood Cells

1. Fill six 15 ml centrifuge tubes with 10 ml of PBS buffer.
2. Using a glass pipette, remove 500 µl of unwashed blood cells from the blood collection tube.
3. Pipette the 500 µl of blood into each of the six tubes containing PBS.
    a. *Pipette up and down continuously until most of the blood has been removed from the side of the pipette walls.*
4. Gently invert every tube several times to make sure the blood is thoroughly mixed into the PBS.
5. Centrifuge the tubes on 313 × *g* for 10 minutes at 4°C.
6. Carefully remove the tubes from the centrifuge without disturbing the packed red blood cells (RBCs).
7. Carefully decant the PBS without pouring out the RBCs.
8. Pour PBS back into the tube to the 10 ml line.

Author Manuscript

This article is protected by copyright. All rights reserved.

9. Gently invert the tubes several times to mix the RBC pellet in the buffer and place them back in the centrifuge.
10. Repeat steps 5-9 four more times.
11. On the final wash, remove the PBS from the tubes using the glass pipette. Do NOT disturb the packed RBCs.
    a. *Leave a very small volume of PBS on top of the pellet to make sure the RBCs don't dry out.*
12. Store the washed RBCs at 4°C.

2. (b) Making a 2% v/v RBC Suspension

13. Fill a 15 ml centrifuge tube with 9.8 ml of PBS buffer.
14. Remove one tube of the packed RBCs from 4°C.
15. Pipette 200 µl of packed RBCs into the 15 ml centrifuge tube using a micropipette.
    a. *Pipette up and down several times until there is no longer any RBCs adhering to wall of the pipette tip.*
16. Invert the centrifuge tube several times and store at 4°C.

Basic Protocol 3

Detection of Lectin Activity of the Crude Extracts

This protocol describes the procedures for detecting lectin activity in the crude solution. The purpose of this procedure is to confirm the presence of lectins in the crude preparations. The presence of lectins in any sample could be determined by a simple and powerful test called hemagglutination assay. Most lectins bind to the surface glycans of RBC plasma membrane and thus form a cross-linked network of RBCs. This is called hemagglutination. When this happens, the RBCs

This article is protected by copyright. All rights reserved.

Author Manuscript

are entangled in the network and cannot settle at the bottom of the microtiter plate, in which the assay is performed. Hemagglutinated RBCs form a distinct pattern (Figure 4) that is easy to detect by visual inspection without the help of any equipment, such as microscopes and plate readers. By using a serially diluted sample, the hemagglutination assay determines the level of lectin activity of that sample. This assay not only detects lectin activity, it is also employed to determine the ligand binding specificity of the lectins.

[*Place figure 4 near here.]

Materials:

2% v/v RBC suspension (generated in Basic Protocol 2)

Micropipette

Micropipette tips

20 mM PBS buffer (see recipe in **Reagents and Solutions**)

96 well U-bottom microtiter plates (ThermoFisher Scientific, Cat #262162)

Crude extract of human Galectin-3 (see Alternate Protocol 3)

Crude extract of Jack bean and Jack bean meal (see Alternate Protocol 1)

Crude extract of soy flour (see Basic Protocol 1)

Crude extract of *A. sativum* agglutinin (see Alternate Protocol 2)

Crude extract of *C. esculenta* lectin (see Alternate Protocol 2)

Crude extract of *X. sagittifolium* lectin (see Alternate Protocol 2)

Author Manuscript

This article is protected by copyright. All rights reserved.

Procedure

1. Pipette 25 µl of PBS buffer into 13 wells of the 96-well round bottom plate using a micropipette.
2. Serial dilute (two-fold serial dilution) 25 µl of the crude extract up to 12 wells. The range of dilution was 1/256 to 1/4096 of the initial crude.
3. Add 25 µl of 2% v/v RBC suspension to all 13 wells.

   13 wells will provide 12 sample wells and 1 negative control (the buffer that was used for preparing the extract was used for negative control)
4. Gently shake the plate a few times.

   Shake the plate very gently until there aren't any visible smears or lines in the bottom of the well
5. Let the plate sit at room temperature undisturbed for 45-60 minutes.
6. Visualize the absence or presence of lectin activity (agglutination) and note the results. Formation of a button at the bottom of the titer plate wells indicates an absence of lectin activity (agglutination), where as a smear-like profile is the signature of lectin activity (agglutination), as shown in Figure 4. The total numbers of wells that show the sign of lectin activity is called the titer value. For example, if a sample shows activity up to the fifth well, it titer value is 5. The titer value is used for quantification in subsequent experiments.

Basic Protocol 4

Identification of Multivalent Inhibitors as TCAs by Hemagglutination Inhibition Assays

In the present study, targets (lectins and GPs) are captured by target capturing agents (TCAs). Proper selection of TCAs is very crucial for CaRe. Protocol 4 describes how TCAs are identified. CaRe is based on the principle that a target must engage in multivalent binding with its TCAs. Therefore, binding affinity of the targets for selected multivalent ligands (potential TCAs) is determined by hemagglutination inhibition assays. The most commonly tested multivalent ligands included

Author Manuscript

This article is protected by copyright. All rights reserved.

invertase, bovive thryroglobulin, mannan, asialofetuin, chondroitin sulfate A and C (see Welch et al., 2020). A representative inhibition profile is shown in Figure 5. Multivalent ligands that show higher affinity for targets are selected as potential TCAs.

[*Place figure 5 near here.]

Materials:

Multivalent Ligands

2% v/v RBC suspension (see Basic Protocol 2)

20 mM PBS buffer or 100 mM HEPES buffer (see recipe in **Reagents and Solutions**)

96 well U-bottom Nunc brand microtiter plates

Micropipette

Micropipette tips

Procedure

1. Adjust the titer of the target lectin you are trying to test to 2-3 agglutination titers (meaning the target lectin sample should agglutinate up to 2 to 3 wells).
2. Pipette 25 µl of PBS or HEPES buffer into 8 wells of the 96-well round bottom plate using a micropipette.
3. Pipette 25 µl of PBS or HEPES buffer into 2 additional wells of the 96-well round bottom plate using a micropipette.
4. Serial dilute (2-fold serial dilution) 25 µl of the ligand into 7 of the 8 wells.
5. Pipette 25 µl of the ligand into the 8th well.
    a. *This will act as the ligand control. Do NOT serial dilute.*

Author Manuscript

This article is protected by copyright. All rights reserved.

6. Pipette 25 µl of the lectin crude into the 7 wells that contains the serial diluted ligand.
    a. *Do NOT pipette your lectin into the 8th well that contains your ligand control.*
7. Pipette 25 µl of the lectin into the one of the 2 additional wells that contain PBS or HEPES buffer.
    a. *This will act as the lectin control.*
8. Gently shake the plate and let it sit at room temperature for 30-60 minutes.
9. Pipette 25 µl of the 2% v/v RBC suspension into all 10 wells.
10. Pipette 25 µl of the 2% v/v RBC suspension in a well containing only 25 µl of buffer (this well is the RBC and buffer control).
11. Gently shake the plate and let it sit at room temperature for 30-45 minutes.
12. Visualize and note the results.
    a. *The multivalent ligands that show inhibitory activities are selected for the precipitation experiments.*

Basic Protocol 5

Testing the capturing abilities of TCAs by precipitation/turbidity assays

Good affinity for targets is not the only criterion of a multivalent ligand to be qualified as an effective TCA. An ideal multivalent ligand for use with the CaRe system must also be able to cross-link (precipitate) the targets.

Protocol 5 describes how the cross-linking or precipitating abilities of TCAs are determined. When the lectins are the targets, multivalent glycoproteins will be screened as TCAs. If the targets are glycoproteins, purified lectins will be used as TCAs. If performed properly, the targets (lectin or glycoprotein) of interest will precipitate out of solution by binding to the TCAs. The extent of precipitation is monitored by a UV-Vis spectrophotometer at 420 nm (Figure 6). The most effective TCA should be chosen based on its ability to precipitate a substantial amount of target out of solution. This is determined by the OD values (at 420 nm) obtained with respective TCAs. The TCA,

Author Manuscript

This article is protected by copyright. All rights reserved.

that gives maximum OD (generates maximum precipitates) compared with other TCAs on a molar basis, is considered as the most effective TCA.

[*Place figure 6 near here.]

Materials:

Multivalent Ligands that showed higher affinity in Basic Protocol 4

Purified Concanavalin A (ConA) (Millipore-Sigma, Cat # L7647)

Crude extract of human Galectin-3 (see Alternate Protocol 3)

Crude extract of Jack bean (see Alternate Protocol 1)

Crude extract of *A. sativum* agglutinin (see Alternate Protocol 2)

Crude extract of *C. esculenta* lectin (Tarin) (see Alternate Protocol 2)

Crude extract of *X. sagittifolium* lectin (see Alternate Protocol 2)

Crude extract of soy flour (see Basic Protocol 1)

Crude extract of egg white (see Alternate Protocol 4)

20 mM PBS buffer or 100 mM HEPES buffer (see recipe in **Reagents and Solutions**)

Micropipettes

Micropipette tips

Quartz cuvettes

UV-Vis Spectrophotometer

1.5 ml microcentrifuge tubes

Author Manuscript

This article is protected by copyright. All rights reserved.

Kimwipes

5. (1) Preparing the Spectrophotometer

1. Change the absorbance on the spectrophotometer to 420 nm.
2. Pipette 1 ml of buffer into the reference and sample cuvettes.
3. Autozero the spectrophotometer.

5. (2) Precipitation of Target Lectins from the Crude Solution

4. Perform "Preparing the Spectrophotometer" before proceeding with step 5.

5. Remove the sample cuvette from the spectrophotometer and decant the buffer.

6. Add 1 ml of the lectin crude with known hemagglutination titer to the sample cuvette.

7. Place the cuvette into the spectrophotometer.

8. Record the absorbance.

9. Autozero the spectrophotometer.

10. Remove the sample cuvette from the spectrophotometer.

11. Pipette 5 µl of the multivalent ligand (stock solution with known concentration) into the cuvette once.

12. Invert the cuvette once.

13. Use a Kimwipe to wipe the cuvette and place it back into the spectrophotometer.

14. Record the absorbance.

15. Continue steps 8-11 until the curve plateaus and slightly decreases.

    a. *Make a note of the amount of ligand added where it plateaus before it decreases.*

Author Manuscript

This article is protected by copyright. All rights reserved.

16. Decant the mixture in the sample cuvette into a 1.5 ml microcentrifuge tube.

17. Incubate the mixture at room temperature for 1-12 hours (incubation time varies based on the samples. It is decided by checking the OD at different time points).

18. Determine the stoichiometric ratio for the target lectin and multivalent ligand. The concentration value of a ligand that produces the highest OD point with a given crude solution on a precipitation curve is noted. That concentration value of the ligand with regard to the given crude solution is the stoichiometric ratio.

Use the concentration of a ligand at its stoichiometric ratio for the respective lectin crude for ***Basic Protocol 6.***

5. (3) Precipitation of Target Glycoproteins from the Crude Solution

19. Perform "Preparing the Spectrophotometer" before proceeding with step 20.

20. Remove the sample cuvette from the spectrophotometer and decant the buffer.

21. Add 1 ml of purified ConA to the sample cuvette.

22. Place the cuvette into the spectrophotometer.

23. Record the absorbance.

24. Autozero the spectrophotometer.

25. Remove the sample cuvette from the spectrophotometer.

26. Pipette 5 µl of the glycoprotein crude into the cuvette once.

27. Invert the cuvette once, after putting a lid on the cuvette.

28. Use a Kimwipe to wipe the cuvette and place it back into the spectrophotometer.

29. Record the absorbance.

Author Manuscript

This article is protected by copyright. All rights reserved.

30. Continue steps 8-11 until the curve plateaus and slightly decreases.

a. Make a note of the amount of glycoprotein crude added where it plateaus before it decreases.

31. Decant the mixture in the sample cuvette into a 1.5 ml microcentrifuge tube.

32. Incubate the mixture at room temperature for 1-12 hours (incubation time varies based on the samples. It is decided by checking the OD at different time points).

The above steps could be performed by placing crude containing target GPs in cuvette *and adding lectins as TCAs (5 µl at a time). This alteration is done if the former approach does not produce sufficient precipitation.*

Basic Protocol 6

Capturing of targets (lectins and GPs) in the crude extracts by TCAs and separation of the target-TCA complex from other components of the crude extracts

Once the most effective TCAs are determined following the procedures described above, specific TCAs are mixed in proper ratios (as described in Basic Protocol 5(2), step 18 above) with the crude preparation containing the targets. The targets bind to the TCAs and form insoluble cross-linked complexes and the mixture becomes turbid (Figure 7). When centrifuged, these complexes settle as pellets at the bottom of the tubes, leaving the impurities in the supernatant (Figure 8). When the supernatant is discarded, the only material remains in the microfuge tubes is the target-TCA complex.

[*Place figure 7 near here.]

Author Manuscript

This article is protected by copyright. All rights reserved.

[*Place figure 8 near here.]

Materials:

Crude extracts

TCA solutions

20 mM PBS buffer or 100 mM HEPES buffer (see recipe in **Reagents and Solutions**). If any lectin requires metal ions, HEPES is used. Otherwise, PBS is used.

Micropipettes

Micropipette tips

Centrifuge

6. (1) Capturing the targets (lectins or GPs)

1. Capture the target lectin with the proper capturing agent (TCA) as described in Basic Protocol 5(2) above.
   For capturing the targeted GP from the crude, use purified ConA as the TCA.
2. Incubate the mixture for 1-12 hours at 4°C (incubation time varies based on the samples. It is decided by checking the OD at different time points).
3. Centrifuge the mixture at 9469 × *g* for 30 minutes at 4^0 C.
4. Discard the supernatant (in certain cases, the supernatant could be reused and steps 1-3 can be done with it to capture additional amount of target)

6. (2) Washing the Insoluble Precipitate

5. Pipette 1 ml of cold buffer (the same buffer the crude sample and the TCA were in) along the wall of the microcentrifuge tube with the precipitate.

Author Manuscript

This article is protected by copyright. All rights reserved.

6. Centrifuge the precipitate with the buffer on 6339 x g for 5 minutes at 4^0 C.

7. Discard the buffer.

8. Repeat steps 5-7 two more times.

After the last wash, keep a small amount of buffer on top of the precipitate to keep the sample from drying.

9. Store at 4^0 C (depending on the samples, they remain stable up to 6 months).

Basic Protocol 7

Releasing the captured targets (lectins and GPs) by dissolving the complex

This protocol presents the procedure for releasing the captured targets (lectins or GPs) from their respective TCAs. This is accomplished by dissolving the target-TCA complex with the help of specific monovalent ligands in appropriate concentration (200 mM to 400 mM, which is about 100-500 times more than respective TCA concentration. Such higher concentrations of monovalent ligands are used to assure a complete dissociation of target-TCA complex. This concentration is determined from dissociation experiments by using a range of concentration of monovalent ligand to find out the minimum concentration of a monovalent ligand required to completely dissolve the target-TCA complex) or by changing the pH of the solution (in certain cases, it is difficult to dissolve target-TCA complex by adding monovalent ligands because some lectins do not bind to monovalent sugars. For those target lectins the target-TCA complex is dissolved by changing the pH of the buffer. Depending on the specificity of the target or captor lectins, mannose, galactose, lactose, fucose and GalNAc could be used as competitive monovalent ligands to dissolve target-TCA complex).

Author Manuscript

This article is protected by copyright. All rights reserved.

Materials:

Precipitated complexes (see Basic Protocol 6)

Monovalent saccharides

20 mM PBS buffer or 100 mM HEPES buffer (see recipe in **Reagents and Solutions**)

100 mM sodium acetate buffer without ions (pH 4.9, see recipe in **Reagents and Solutions**)

Micropipettes

Micropipette tips

Procedure

1. Make a high concentration (200 mM-400mM) of monovalent saccharides in PBS.
2. Remove the recently precipitated complexes from 4^0 C (from Basic Protocol 6).
3. Pipette 1 ml of the monovalent saccharide solution along the wall of the microcentrifuge tube containing the complex.
4. Incubate the mixture for 4-18 hours until the complex dissolves and the opaque solution becomes gradually clear (checked visually, can also be checked by measuring OD at 420 nm). For most protein tested, one can vortex the mixture to dissolve the precipitate quickly.
5. If the complex does not dissolve, perform the following steps:
    a. Centrifuge the mixture on 6339 x g for 15 minutes at 4^0 C.
    b. Pipette 1 ml of cold buffer along the wall of the microcentrifuge tube holding the precipitate.
    c. Centrifuge the precipitate with the buffer on 6339 x g for 5 minutes at 4^0 C.
    d. Discard the buffer.
    e. Pipette 1 ml of 100 mM sodium acetate buffer (pH 4.9) into the microcentrifuge tube.
    f. Incubate the solution for 4-18 hours.
    g. Vortex the solution if some precipitate is still observed.

Author Manuscript

This article is protected by copyright. All rights reserved.

Basic Protocol 8

Separation of the targets (lectins and glycoproteins) from their respective TCAs

The purpose of this procedure describe in this protocol is to separate the targets from the TCAs by filtration or chromatography. As described above, the main features of an effective TCA are their affinity for their targets as well as their ability to precipitate the targets. As mentioned in the Strategic Planning above, an essential factor to consider during the selection of TCAs is their molecular weight. The molecular weights of the TCAs should differ from their targets (at least by 10 kDa). This is necessary for proper separation of the targets from their TCAs.

Materials:

Dissociated target-TCA complexes (from Basic Protocol 7)

Amicon membrane filtration tubes MWCO: 100 kDa, 50 kDa, 30 kDa, 10 kDa (Millipore-Sigma, Catalog #: UFC9100, UFC9050, UFC9030, UFC9010)

20 mM PBS Buffer (see recipe in **Reagents and Solutions**)

100 mM sodium acetate buffer (pH 5.2 without ions, see recipe in **Reagents and Solutions**)

Flex-Column Economy Column 177 × 1.5 cm (Kimble-Chase, Catalog #420401-1511)

Fraction collection tubes

Bio-gel P-100 beads (Bio-Rad, Catalog #1504170)

Micropipette

Micropipette tips

Centrifuge

Quartz cuvettes

UV-vis spectrophotometer

Author Manuscript

This article is protected by copyright. All rights reserved.

8. (1) Separating the Galectin-3-TCA complexes

1. Wash the membrane filtration tubes per the manufacturer's instructions.
2. Pipette the dissociated complex onto the 100 kDa or 50 kDa molecular weight cutoff membrane filtration tube.

   Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands (TCAS) and their relative differences from those of the targets. For example, bovine thyroglobulin (Tg), the TCA of Galectin-3 is 670 kDa, whereas the molecular weight of the target (Galectin-3) is 29 kDa (monomeric) and 150 kDa (oligomeric).
3. Centrifuge the mixtures on 5000 x g for 10 minutes 4^0 C.
4. Remove the filter from the centrifuge.
5. Pipette the bottom fraction onto a 10 kDa membrane filtration tube.
6. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.
7. Remove the filter from the centrifuge.
8. Pipette 5 ml of PBS buffer onto the top of the filter.
9. Centrifuge the membrane filtration tube until the final volume on the top of the filter is approximately 1 ml.
10. Use this fraction to check the purity of the lectin by SDS-PAGE.
11. The sample at the bottom of the filter is also checked by SDS-PAGE.

8. (2) Separating the ConA-TCA complexes

12. Wash the membrane filtration tubes per the manufacturer's instructions.
13. Pipette the dissociated complex onto the 100 kDa membrane filtration tube.

    a. *Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.*
14. Centrifuge the mixtures on 5000 x g for 10 minutes 4^0 C.

Author Manuscript

This article is protected by copyright. All rights reserved.

15. Remove the filter from the centrifuge.

16. Pipette the bottom fraction onto a 50 kDa membrane filtration tube.

17. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.

18. Remove the filter from the centrifuge.

19. Pipette 5 ml of HEPES buffer onto the top of the filter.

20 Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.

21. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (3) Separating the SBA-TCA (non-ConA) complexes

22. Wash the membrane filtration tubes per the manufacturer's instructions.

23. Pipette the dissociated complex onto the 100 kDa membrane filtration tube.

Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.

24. Centrifuge the mixtures on 5000 x g for 10 minutes 4^0 C.

25. Remove the filter from the centrifuge.

26. Pipette 5 ml of PBS buffer onto the top of the filter.

27. Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.

28. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (4) Separating the ASA-TCA complexes

29. Wash the membrane filtration tubes per the manufacturer's instructions.

Author Manuscript

This article is protected by copyright. All rights reserved.

30. Pipette the dissociated complex onto the 100 kDa membrane filtration tube.

Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.

31. Centrifuge the mixtures on 5000 x g for 10 minutes 4^0 C.

32. Remove the filter from the centrifuge.

33.Pipette the bottom fraction onto a 50 kDa membrane filtration tube.

34. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.

35. Remove the filter from the centrifuge.

36. Pipette 5 ml of HEPES buffer onto the top of the filter.

37. Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.

38. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (5) Separating the C. esculenta lectin-TCA complexes

39. Wash the membrane filtration tubes per the manufacturer's instructions.

40. Pipette the dissociated complex onto the 100 kDa membrane filtration tube.

a. Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.

41. Centrifuge the mixtures on 5000 x g for 10 minutes 4^0 C.

42. Remove the filter from the centrifuge.

43. Pipette the bottom fraction onto a 50 kDa membrane filtration tube.

44. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.

45. Remove the filter from the centrifuge.

Author Manuscript

This article is protected by copyright. All rights reserved.

46. Pipette 5 ml of HEPES buffer onto the top of the filter.

47. Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.

48. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (6) Separating the X. sagittifolium lectin-TCA complexes

49. Wash the membrane filtration tubes per the manufacturer's instructions.

50. Pipette the dissociated complex onto the 100 kDa membrane filtration tube.

    a. *Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.*

51. Centrifuge the mixtures on 5000 x g for 10 minutes 4^0 C.

52. Remove the filter from the centrifuge.

53. Pipette the bottom fraction onto a 50 kDa membrane filtration tube.

54. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.

55. Remove the filter from the centrifuge.

56. Pipette 5 ml of HEPES buffer onto the top of the filter.

57. Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.

58. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (7) Separating the Ovalbumin-ConA complexes

59. Wash the membrane filtration tubes per the manufacturer's instructions.

60. Pipette the dissociated complex onto the 100 kDa membrane filtration tube.

Author Manuscript

This article is protected by copyright. All rights reserved.

a. *Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.*

61. Centrifuge the mixture on 5000 x g for 10 minutes 4^0 C.
62. Remove the filter from the centrifuge.
63. Pipette the bottom fraction onto a 50 kDa membrane filtration tube.
64. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.
65. Remove the filter from the centrifuge.
66. Pipette the bottom fraction onto a 30 kDa membrane filtration tube.
67. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.
68. Remove the filter from the centrifuge.
69. Pipette 5 ml of PBS buffer onto the top of the filter.
70. Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.
71. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (8) Separating the Tarin-ConA complexes

72. Wash the membrane filtration tubes per the manufacturer's instructions.
73. Pipette the dissociated complex onto the 30 kDa membrane filtration tube.
    a. *Molecular weight cutoff of the filters is selected based on the masses of the multivalent ligands and their relative differences from those of the targets.*
74. Centrifuge the mixture on 5000 x g for 10 minutes 4^0 C.
75. Remove the filter from the centrifuge.

Author Manuscript

This article is protected by copyright. All rights reserved.

76. Pipette the bottom fraction onto a 10 kDa membrane filtration tube.

77. Centrifuge the fraction on 5000 x g for 10 minutes 4^0 C.

78. Remove the filter from the centrifuge.

79. Pipette 5 ml of sodium acetate buffer onto the top of the filter.

80. Centrifuge the membrane filtration tube until the final volume is approximately 1 ml.

81. Use this fraction to check the purity of the lectin using SDS-PAGE.

8. (9) Separating the SBA-ConA complexes

82. Prepare and calibrate a Flex-Column Economy Column (dimensions: 177 × 1.5 cm) with Bio-gel P-100 beads according to the manufacturer's instructions.

83. Set the flow rate for the column.

84. Load approximately 1 ml of the dissociated complex onto the column.

85. When the complex has been absorbed onto the column pipette 100 mM sodium acetate (pH 5.0 to5.2) buffer onto the column.

86. Use 100 mM sodium acetate buffer as the mobile phase for the column.

87. Collect the fractions.

88. Check each fraction using a UV-vis spectrophotometer at an absorbance of 280 nm.

89. Check the purity of the fractions using SDS-PAGE.

Author Manuscript

This article is protected by copyright. All rights reserved.

Basic Protocol 9

Verification of the purity of the isolated targets (lectins or GPs)

After the separation from their TCAs, the targets need to be checked for their purity. This is done by SDS-PAGE. A representative SDS-PAGE profile is shown in Figure 9.

[*Place figure 9 near here.]

Materials:

SDS-PAGE running buffer (see recipe in **Reagents and Solutions**)

SDS-PAGE sample buffer (see recipe in **Reagents and Solutions**)

SDS-PAGE staining solution (see recipe in **Reagents and Solutions**)

SDS-PAGE de-staining solution (see recipe in **Reagents and Solutions**)

TGX Stain-free gels 12% (Bio-Rad, Catalog #4568043)

Broad range prestained protein standard (New England Biolabs, Catalog #P7719S)

0.6 ml microcentrifuge tubes

Micropipettes

Micropipette tips

100 ml beaker

Erlenmeyer Flask

Author Manuscript

This article is protected by copyright. All rights reserved.

Glass Pipettes

Gel loading tips

Mini Protein Tetra cell (BIO-RAD)

PowerPac Power Supply (BIO-RAD)

Microcentrifuge compatible heater (Themo-Fisher)

Procedure

1. Remove SDS-PAGE running buffer from 4^0 C.
2. Turn the heater on and set the temperature to 95^0 C.
3. Make a 1X solution of the SDS-PAGE running buffer.
4. Decant 50 ml of the 1X running buffer into a 100 ml beaker.
5. Remove the TGX stain-free gels from 4^0 C and wash them with the 100 ml of 1X running buffer according to the manufacturer's instructions.
6. Set up the Mini-Protein Tetra cell according to the manufacturer's instructions.
7. Remove the protein standard from -20^0 C and bring it to room temperature.
8. Pipette 950 µl of sample buffer into a 1.5 ml microcentrifuge tube.
9. Pipette 50 µl of β-mercaptoethanol into the sample buffer.
    a. *Mix the solution thoroughly by pipetting up and down or vortex.*
10. Pipette 20 µl of each sample into the 0.6 ml microcentrifuge tubes.
    a. *Include commercially available purified samples as references.*
11. Pipette 40 µl of the sample buffer into each sample tube
    a. *Mix thoroughly by pipetting up and down.*
12. Place the tube in the heater set for 95^0 C.
13. Let the tubes incubate at 95^0 C for 5 minutes.
14. Remove the tubes from the heater.
15. Pipette 10 µl of the protein standard into one of the wells on the gel.
16. Pipette 20 µl of each sample into the wells of the gel.
17. Run the gel at 150 V until finished (the dye reaches the bottom of the gel).

Author Manuscript

This article is protected by copyright. All rights reserved.

18. Stain the gel for 2 hours using enough SDS-PAGE staining solution to cover the gel.
19. Discard the staining solution after staining.
20. Add the de-staining solution to cover the gel and let the gel de-stain until the desired background is achieved.
21. Note the results.

REAGENTS AND SOLUTIONS

Use ultrapure or deionized water in all recipes. All SDS-PAGE recipes were taken from a manual provided by the manufacturer.

1. 20 mM Phosphate Buffered Saline, pH 7.4 (10X)

1. Weigh out 4.83 g of sodium phosphate monobasic monohydrate and place it in a 1 L beaker with a stir bar.
2. Add 200 ml of ultrapure water and begin to dissolve the solute.
3. Weigh out 42.88 g of sodium phosphate dibasic heptahydrate and add it to the 1 L beaker.
4. Add an additional 300 ml of ultrapure water.
5. Let the solute completely dissolve before performing the next step.
6. Weigh out 90.0 g of sodium chloride and add it to the 1 L beaker.
7. Add water to the 900 ml mark.
8. Let the solute completely dissolve and then transfer the solution to a 1 L graduated cylinder.
9. Adjust the volume to 1 L, cover, and invert several times.
10. Measure the pH of the solution (pH should be 6.7-6.8).
11. Make a 1X dilution from the stock and check the pH (pH should be 7.3-7.4).
12. Store the 10X and 1X at room temperature until ready for use.

2. 100 mM HEPES Buffer with calcium and manganese, pH 7.3 (1X)

1. Weigh out 23.88 g of HEPES and place it in a 1 L beaker with a stir bar.

Author Manuscript

This article is protected by copyright. All rights reserved.

2. Add 150 ml of ultrapure water and begin to dissolve the solute.
3. Weigh out 0.74 g of calcium chloride and add it to the 1 L beaker.
4. Add an additional 150 ml of ultrapure water and continue to dissolve the solute.
5. Weigh out 8.77 g of sodium chloride and fill the beaker to the 450 ml mark.
6. Let the solute completely dissolve.
7. Make a 2M NaOH solution.
8. When finished, adjust the pH of the 1X HEPES buffer to 7.2.
9. Weigh out 0.99 g of manganese chloride and add it to the 1 L beaker.
10. Let the solute completely dissolve and then transfer the solution to a 1 L graduated cylinder.
11. Adjust the volume to 1 L and check the pH again (pH should be 7.2-7.3).
12. Store the 1X solution at room temperature until ready for use.

3. 100 mM Sodium Acetate with calcium and manganese, pH 5.2 (1X)

1. Weigh out 13.6 g of sodium acetate and place it in a 1 L beaker with a stir bar.
2. Add 200 ml of ultrapure water and begin to dissolve the solute.
3. Weigh out 0.74 g of calcium chloride and add it to the 1 L beaker.
4. Add an additional 150 ml of ultrapure water and continue to dissolve the solute.
5. Weigh out 8.77 g of sodium chloride and fill the beaker to the 450 ml mark.
6. Let the solute completely dissolve.
7. Check the pH.
8. Adjust the pH to 5.2 using acetic acid.
9. Once the desired pH has been reached, adjust the volume to 950 ml and let the solution mix.
10. Weigh out 0.99 g of manganese chloride and add it to the 1 L beaker.
11. Let the solute completely dissolve and then transfer the solution to a 1 L graduated cylinder.
12. Adjust the volume to 1 L and check the pH again (pH should be 5.1-5.2).
13. Store the 1X solution at room temperature until ready for use.

4. 100 mM Sodium Acetate without calcium and manganese, pH 5.2 (1X)

1. Weigh out 13.6 g of sodium acetate and place it in a 1 L beaker with a stir bar.

Author Manuscript

This article is protected by copyright. All rights reserved.

2. Add 950 ml of ultrapure water.
3. Let the solute completely dissolve.
4. Check the pH.
5. Adjust the pH to 5.2 using acetic acid.
6. Adjust the volume to 1 L and check the pH again (pH should be 5.1-5.2).
7. Store the 1X solution at room temperature until ready for use.
    a. *Note: For the sodium acetate pH 4.9 solution, follow the same steps but adjust the pH to 4.9*.

5. Tris-HCl buffer with EDTA, pH 7.5

Steps for 1 M Tris-HCl 500 ml solution:

1. Dissolve 60.5 g of trizma base into 350 ml of ultrapure water.
2. Adjust pH to 7.85 by adding 12.1 N HCl and monitor the change in pH using a pH meter.
3. Adjust the final volume to 500 ml.
4. The pH should change to ~7.6.
5. Store the stock in the refrigerator after use.

How to 22 mM Tris-HCl EDTA

1. Take 22 ml of the 1 M Tris-HCl and add it to 978 ml of ultrapure water.
2. The pH should be around 7.73.
3. Weigh out 1.86 g of EDTA and add it to the solution.
4. Let the EDTA completely dissolve.
5. Measure the pH using the pH meter.
6. Adjust with NaOH or HCl as needed.

Author Manuscript

This article is protected by copyright. All rights reserved.

6. SDS-PAGE Running Buffer (10X)

1. Weigh out 30.3 g of Tris base and place it in a 1 L beaker with a stir bar.
2. Add 200 ml of ultrapure water
3. Weigh out 144.1 g of Glycine and place it in the 1 L beaker
4. Adjust the volume in the beaker to 600 ml by adding 400 ml of ultrapure water
5. Weigh out 10.0 g of sodium dodecyl sulfate (SDS) and place it in the 1 L beaker
6. Adjust the volume in the beaker to 900 ml in 300 ml of ultrapure
7. Let the solute completely dissolve and then transfer the solution to a 1 L graduated cylinder.
8. Adjust the volume to 1 L and invert several times to thoroughly mix.
9. Store the solution at 4°C.
    a. *Note: when ready for use make a 1 X solution to use as the running buffer. Store any excess buffer at 4°C.*

7. SDS-PAGE Staining Solution

1. Add 175 ml of ultrapure water to a 250 ml graduated cylinder.
2. Adjust the volume of the cylinder to 225 ml by adding 50 ml of methanol.
3. Adjust the volume of the cylinder to 250 ml by adding 25 ml of acetic acid.
4. Invert the graduated cylinder several times to mix.
5. Weigh out 0.625 g of Coomassie brilliant blue.
6. Dissolve the powder into the solution in an Erlenmeyer flask.
7. Filter the solution to remove any undissolved powder.
8. Store at room temperature until ready for use.

8. SDS-PAGE Sample Buffer

Mix the following ingredients in a 50 ml falcon tube

1. 3.75 ml of 0.5 M Tris-HCl pH 6.8
2. 15 ml of 50% Glycerol

Author Manuscript

This article is protected by copyright. All rights reserved.

3. 0.3 ml of 1.0% Bromophenol blue
4. 6 ml of 10% SDS
5. Adjust volume to 30 ml by adding ultrapure water.
6. Store the solution at room temperature until ready for use.

9. SDS-PAGE Destaining Solution

1. Take out a 500 ml graduated cylinder.
2. Add 375 ml of ultrapure water to the cylinder.
3. Carefully add 75 ml of methanol to the cylinder.
4. Carefully add 50 ml of acetic acid to the cylinder.
5. Invert the graduated cylinder several times to mix and store the solution at room temperature until ready for use.

COMMENTARY

BACKGROUND INFORMATION:

Column chromatographic techniques (affinity, size exclusion, ion exchange) for protein (including lectins and glycoproteins) purification are time intensive, elaborate, expensive, multistep, and they often co-purify undesirable proteins. Immunoprecipitation (IP), another widely used technique for protein identification/purification, requires antibodies against the targets and substantial amount of prior knowledge about the targets. IP is also associated with antibody contamination, non-specific binding and sample degradation. The present method (CaRe) was formulated to address some of these issues. CaRe was successfully employed to purify several lectins and glycoproteins (see Welch et al., 2020). CaRe is faster and cheaper. This method does not require expensive affinity matrix, antibody, elaborate infrastructure, controlled environment, specialized equipment and functionalization of reagents.

Author Manuscript

This article is protected by copyright. All rights reserved.

CRITICAL PARAMETERS:

The most important part of CaRe is the selection of TCAs. The success of CaRe depends of the following conditions. Binding of the target to the TCA must be multivalent and the binding should lead to precipitation formation (through non-covalent cross-linking). The precipitates should dissolve by specific monovalent ligands or by changing the pH. The molecular weight of the targets should not be close to that of the TCAs to ensure proper separation of purified targets from their TCAs. Most lectins show hemagglutination activities. Therefore, the presence of a lectin in a crude solution can easily be done by a simple agglutination test. This test is also crucial for the identification of TCAs. Some lectins, however, do not show hemagglutination activity. For those lectins, TCAs could be identified by precipitation assays as described above in this protocol.

TROUBLESHOOTING:

It is not common but sometimes the precipitates are not easy to dissolve. However, this problem is solved by altering the concentration of the competitive monovalent ligands, increasing the incubation time with the monovalent ligands or changing the pH of the solution. As mentioned above, TCAs with molecular weights similar to those of the targets should not be used in this method. If their molecular weights are close, a combination of membrane filtration, gel filtration and SDS-PAGE analysis can be used to separately identify them. If the amount of precipitation is insufficient, the crude should be concentrated and the incubation time with the TCAs should be increased.

Author Manuscript

This article is protected by copyright. All rights reserved.

UNDERSTANDING RESULTS:

Results obtained at each step of CaRe are easily understandable. For example, hemagglutination patterns (Figure 4) indicate lectin activity in crude solution. Inhibition patents and precipitation curves (Figure 5, Figure 6) help identify TCAs, Formation of precipitates represents target capturing by TCAs (Figure 7, Figure 8). SDS-PAGE profiles prove proper separation of targets from their respective TCAs as well as the purity of the targets (Figure 9).

TIME CONSIDERATIONS:

Basic Protocol 1: Preparation of Crude Extracts containing the target proteins: 12-72 hours.

Basic Protocol 2: Preparation of 2% v/v Red Blood Cell (RBC) Suspension (2 hours).

Basic Protocol 3: Detection of Lectin Activity in the Crude Extracts (1 hour).

Basic Protocol 4: Identification of Multivalent Inhibitors as TCAs by Hemagglutination Inhibition Assays (2 hours).

Basic Protocol 5: Testing the capturing abilities of TCAs by precipitation/turbidity assays (45 minutes per ligand).

Author Manuscript

This article is protected by copyright. All rights reserved.

Basic Protocol 6: Capturing of targets (lectins and glycoproteins) in the crude extracts by TCAs and separation of the target-TCA complex from other components of the crude extracts (1 hour to 12 hours).

Basic Protocol 7: Releasing the captured targets (lectins and glycoproteins) by dissolving the complex (30 minutes to 12 hours)

Basic Protocol 8: Separation of the targets (lectins and glycoproteins) from their respective TCAs (1 hour to 4 hours).

Basic Protocol 9: Verification of the purity of the isolated targets (lectins or glycoproteins) (2 hours).

Approximate total time required to optimize TCAs is 3 to 4 hours. Approximate time to perform the entire assay following TCA determination is 4 to 12 hours.

ACKNOWLEDGEMENTS:

This work was supported by National Science Foundation Grant 1608537 (to T.K.D.). A part of this work was supported by a startup fund (to P.B.) and by the Research Excellence Fund (to T.K.D.) provided by Michigan Technological University.

Author Manuscript

This article is protected by copyright. All rights reserved.

LITERATURE CITED:

1. Stanley P, Taniguchi N, Aebi M. N-Glycans. 2017. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.

2. Brockhausen I, Stanley P. O-GalNAc Glycans. 2017. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.

3. Lindahl U, Couchman J, Kimata K, Esko JD Proteoglycans and Sulfated Glycosaminoglycans. 2017. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.

6. Varki, A. 2017. Biological roles of glycans. Glycobiology 27:3–49.

4. Taylor ME, Drickamer K, Schnaar RL, Etzler, M. E., and Varki, A. Discovery and Classification of Glycan-Binding Proteins. 2017. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.

5. Esko JD, H. Prestegard J, Linhardt RJ. Proteins That Bind Sulfated Glycosaminoglycans. 2017. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.

Author Manuscript

This article is protected by copyright. All rights reserved.

7. Arora, S., Saxena,V., and Ayyar, B.V. 2017. Affinity chromatography: a versatile technique for antibody purification. Methods 116:84–94.

8. Burgess, R.R. 2018. A brief practical review of size exclusion chromatography: rules of thumb, limitations, and troubleshooting. Protein Expr. Purif. 150:81–85.

9. Cummins, P.M., Rochfort, K.D., and O'Connor, B.F. 2017. Ion-exchange chromatography: basic principles and application. Methods Mol. Biol. 1485:209–223.

10. Speth, C., Toledo-Filho, L.A., and Laubinger, S. 2014. Immunoprecipitation-based analysis of protein-protein interactions. Methods Mol. Biol. 1158:175–185.

11. Lin, J.S., and Lai, E.M. 2017. Protein-protein interactions: co-immunoprecipitation. Methods Mol. Biol. 1615:211–219.

12. Kaboord, B., and Perr, M. 2008. Isolation of proteins and protein complexes by immunoprecipitation. Methods Mol. Biol. 424:349–364.

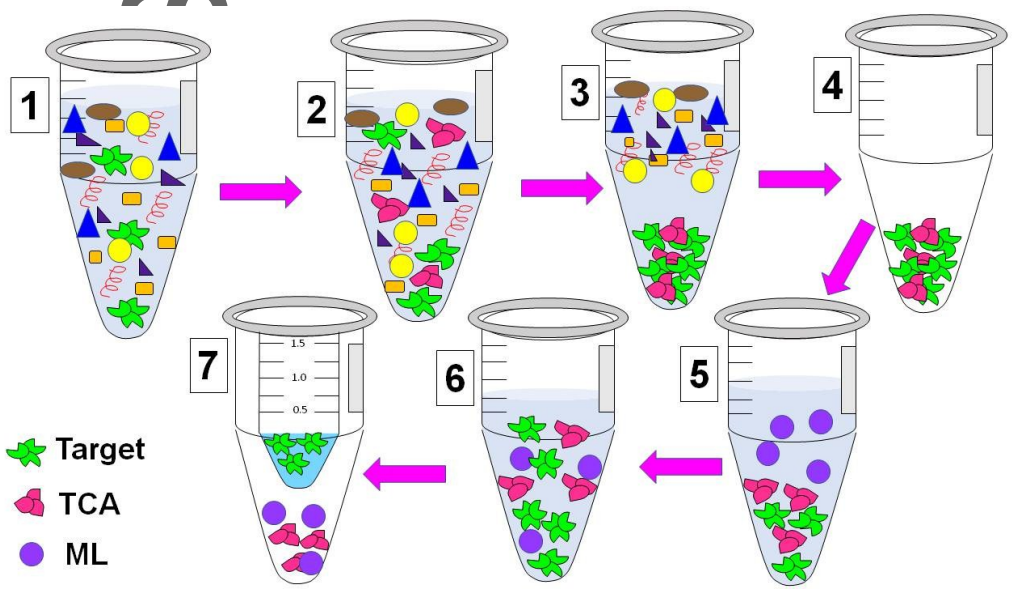
13. Welch C.J, Talaga M.L, Kadav P.D, Edwards J.L, Bandyopadhyay P, Dam T.K. 2020. A capture and release method based on noncovalent ligand cross-linking and facile filtration for purification of lectins and glycoproteins. J. Biol. Chem. 295:223-236.

Author Manuscript

This article is protected by copyright. All rights reserved.

FIGURE LEGENDS:

Figure 1. Schematic presentation of the Capture and Release (CaRe) method. **1**, crude extracts. **2**, crude extracts mixed with a target capturing agent (TCA). **3**, TCA captures target (lectin or glycoprotein) by binding and cross-linking the target. **4**, after centrifugation, the supernatant containing all the impurities are discarded. Only the target-TCA complex remains in the test tube. **5**, monovalent ligand (ML) is added to the complex. **6**, ML dissolves the complex and releases the target. **7**, the released target is separated from the TCA by filtration.



Author Manuscript

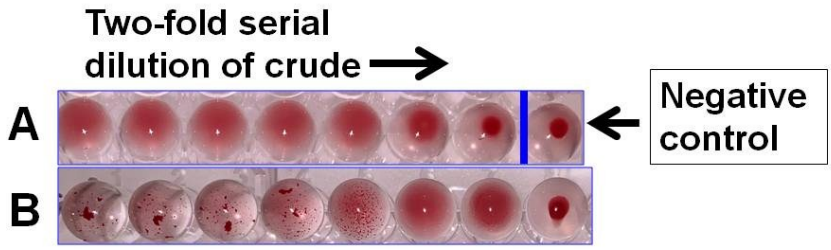
This article is protected by copyright. All rights reserved.

Figure 2. Crude extract of Jack bean.

Figure 3. 2% v/v rabbit RBC in PBS.

This article is protected by copyright. All rights reserved.

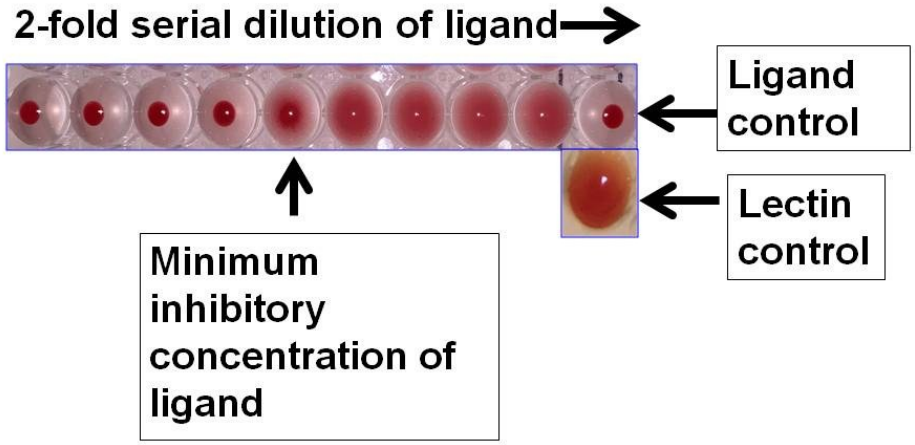
Figure 4. Detection of lectin activity of the crude extracts by hemagglutination assay. A, hemagglutination by Jack bean extract. B, hemagglutination by crude extract containing Gal-3. A two-fold serial dilution method was used to dilute the crude extracts for assaying their lectin activity. The sample is diluted from the left to right side of the plate. A single dot at the bottom of the titer plate well (formed by the accumulation of non-agglutinated RBCs), as observed with the negative control, indicates the absence of lectin activity. A smear or coagulation-like appearance represents lectin activity, which is expressed as "titer value". Titer value is the total number of wells that show lectin activity. For a purified lectin, this activity can be expressed as "specific activity" with regard to the lectin concentration. This is not possible for crude, because it is impossible to determine the concentration of a lectin in a crude solution that contains other proteins. Therefore, "titer value" is the working unit for crude solutions.



Manuscript
Aut

This article is protected by copyright. All rights reserved.

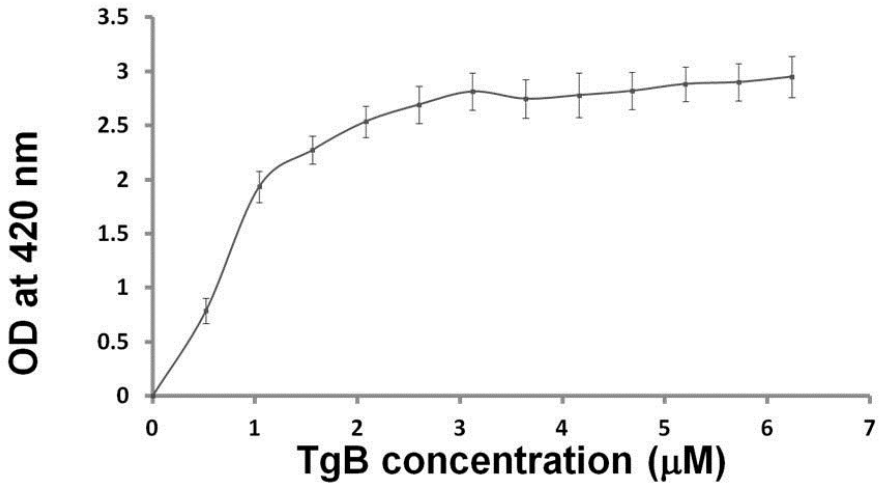
Figure 5. Selection of target capturing agents by inhibition tests. Inhibition of lectin activity of Gal-3 by bovine thyroglobulin (Tg).



Author M

This article is protected by copyright. All rights reserved.

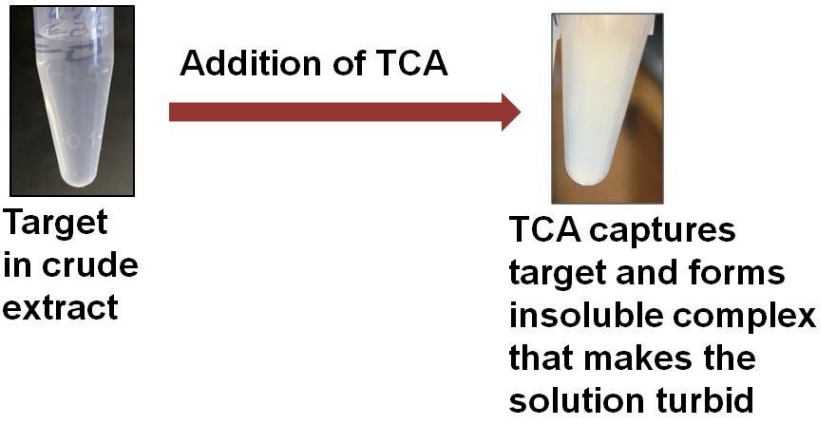
Figure 6. Determining the target capturing ability of a TCA. The efficiency of a TCA (bovine thyroglobulin, in this case) to capture a target (Gal-3, in this case) is proportional to the extent of precipitation formation. The increasing amount of precipitation was documented by a spectrophotometer at 420 nm. The concentration of the TCA that gave the ratio (with regard to the crude) to reach the plateau on the precipitation curve was used in the subsequent CaRe experiments.



Author

This article is protected by copyright. All rights reserved.

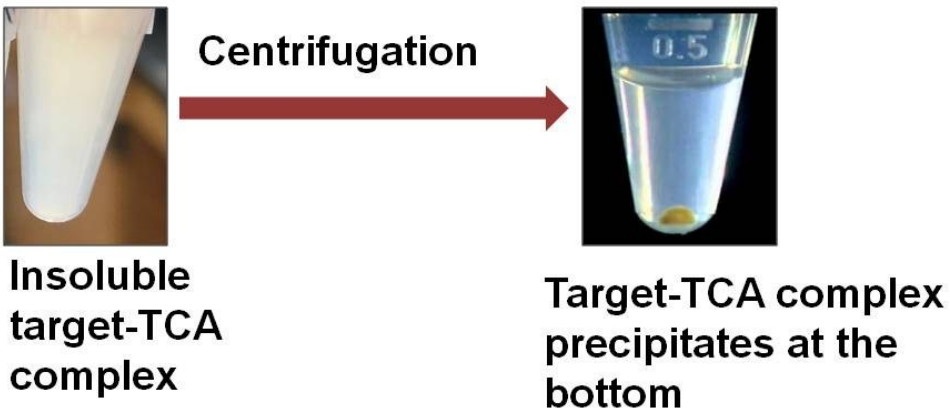
Figure 7. Capturing of targets by TCAs and visualization of insoluble target-TCA complex. An example is shown with Gal-3 as a target and thyroglobulin (Tg) as the TCA. Tg binds and cross-link Gal-3 and forms insoluble complex. The solution becomes turbid due to the formation of target-TCA complex.



Author M

This article is protected by copyright. All rights reserved.

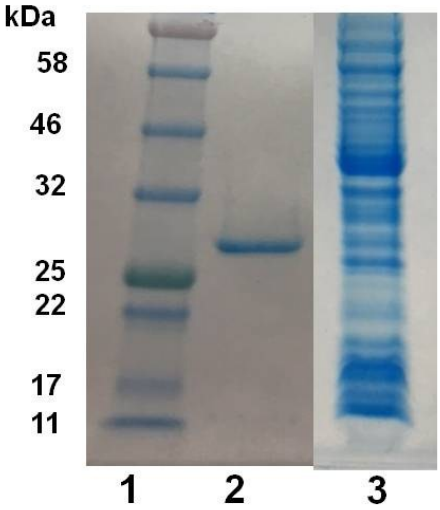
Figure 8. Separation of target-TCA complex from other components of the crude solution. The complex is separated from other impurities of the crude by centrifuging the mixture. The complex containing only the target and the TCA is settled at the bottom of the microfuge tube. The supernatant containing the remaining components of the crude extract is discarded. The precipitate is washed with buffer and then dissolved by competitive monovalent ligands. Thus the target captured with TCA is released. The released target is separated from the TCA by filtration to get the target in pure form.



Auth
Script

This article is protected by copyright. All rights reserved.

Figure 9. Confirmation of the purity of the separated target by SDS-PAGE. An example of purification by CaRe is shown by using Gal-3 after separating it from its TCA (Tg). The profile shows molecular weight markers (Lane 1), Gal-3 purified by CaRe method (lane 2). A profile of the crude extract containing Gal-3 (lane 3) is shown to highlight the extent of purification.



Author

This article is protected by copyright. All rights reserved.