A capture and release method based on noncovalent ligand cross-linking and facile filtration for purification of lectins and glycoproteins

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Glycan-binding proteins such as lectins are ubiquitous proteins that mediate many biological functions. To study their various biological activities and structure–function relationships, researchers must use lectins in their purest form. Conventional purification techniques, especially affinity column chromatography, have been instrumental in isolating numerous lectins and glycoproteins. These approaches, however, are time-consuming, consist of multiple steps, and often require extensive trial-and-error experimentation. Therefore, techniques that are relatively rapid and facile are needed. Here we describe such a technique, called capture and release (CaRe). The strength of this approach is rooted in its simplicity and accuracy. CaRe purifies lectins by utilizing their ability to form spontaneous noncovalently cross-linked complexes with specific multivalent ligands. The lectins are captured in the solution phase by multivalent capturing agents, released by competitive monovalent ligands, and then separated by filtration. CaRe does not require antibodies, solid affinity matrices, specialized detectors, a customized apparatus, controlled environments, or functionalization or covalent modification of reagents. CaRe is a time-saving procedure that can purify lectins even from a few milliliters of crude protein extracts. We validated CaRe by purifying recombinant human galectin-3 and five other known lectins and also tested CaRe’s ability to purify glycoproteins. Besides purifying lectins and glycoproteins, CaRe has the potential to purify other glycoconjugates, including proteoglycans. This technique could also be used for nonlectin proteins that bind multivalent ligands. Given the ubiquity of glycosylation in nature, we anticipate that CaRe has broad utility.

Lectins or agglutinins belong to a large family of proteins called glycan-binding proteins (1). From viruses to humans, lectins are found in almost all taxonomic groups tested so far (2–6). Lectins have been implicated in a variety of biological functions, including cell signaling, cell trafficking, pathogen invasion, immune defense, and metastasis (7–12).

To characterize the structure–function relationship of lectins and elucidate their biological functions, lectins must be purified to homogeneity. Therefore, lectin purification is a crucial step, and it is one of the most extensively performed operations in biochemical/glycobiological research. Purification of lectins, native and recombinant, can be achieved through a variety of approaches.

Conventional protein purification techniques include affinity column chromatography, ion exchange chromatography, and size exclusion chromatography (13–18). The contributions of these methods to protein science are profound, as they helped discover hundreds of proteins, including lectins. Besides purifying lectins, affinity column chromatography has been used extensively for glycoprotein enrichment and purification (19, 20). Column chromatography—based purification methods, however, have multiple steps and are tedious and time-consuming. Other challenges associated with these widely used techniques include an elaborate infrastructure, high cost, requirement for a larger volume of starting crude samples, and nonspecific binding.

There are other purification/separation techniques that use different tools and strategies, including membranes that are chemically modified with different affinity ligands (21), functionalized magnetic nanoparticles (22), and aqueous two-phase systems (23–26). These approaches produce promising results, but they have their limitations. For example, modification and functionalization of the substrata (membranes and magnetic nanoparticles) are tedious chemical processes involving multiple steps and reagents. Aqueous two-phase systems use specific pairs of reagents (polymer–salt or polymer–polymer). The success of this method depends on time-consuming manipulations of a variety of properties of the reagents through multiple trials and calculations.

To minimize some of the challenges described above, we recently developed a purification method for lectins and glycoproteins. This approach, called capture and release (CaRe), is

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3 The abbreviations used are: CaRe, capture and release; TCA, target-capturing agent; ConA, concanavalin A; SBA, soybean agglutinin; ASA, Allium sativum agglutinin; CSA, chondroitin sulfate A; CSC, chondroitin sulfate C; Tg, thyroglobulin; RBC, red blood cell; ASF, asialofetuin; IP, immunoprecipitation.

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relatively rapid and simple. In this method, target lectins are captured in solution phase by multivalent capturing agents (called target-capturing agents (TCAs). The captured lectins are released from TCAs by competitive monovalent ligands, and then the released lectins are separated from the TCAs by membrane filtration or gel filtration (Fig. 1). This method does not require functionalization or covalent modification of the reagents. CaRe needs minimum infrastructural support and space; it does not need solid affinity matrices, specialized detectors, a customized apparatus, or controlled environments. The method is capable of purifying lectins even from a few milliliters of crude extracts. The procedure can be performed in a few microfuge tubes. Detection, purification, and sequence identification of an unknown lectin can be done in a single day. We purified recombinant human galectin-3 (Gal-3) by employing CaRe and subsequently validated the method by purifying five other known lectins. Besides lectins, CaRe was also employed to purify glycoproteins, such as ovalbumin from chicken egg whites as well as the plant glycoproteins SBA and Tarin. The results are described below (Table 1).

Purification of human galectin-3 by CaRe

Chondroitin sulfate A (CSA), chondroitin sulfate C (CSC), and bovine thyroglobulin (Tg) were efficient agents for capturing Gal-3—Escherichia coli cell lysate containing recombinant human Gal-3 showed strong hemagglutination activity. Inhibition tests were performed using this lysate. Among all saccharides, glycosaminoglycans, and glycoproteins tested, lactose, CSA, CSC, and Tg inhibited agglutination of rabbit RBC by the cell lysate. Multivalent ligands are capable of precipitating multivalent lectins through noncovalent, spontaneous, and specific cross-linking. Such ligands could be used as capturing agents for specific lectins. Our previous study (31) showed that CSA and CSC were multivalent ligands of Gal-3. Tg contains 18 complex-type glycans per molecule (nine per subunit). Thus, its interaction with Gal-3 is also multivalent. We therefore selected these three ligands as potential TCAs for...
Gal-3. In quantitative precipitation assays (where increasing amounts of ligands were added to a fixed volume of cell lysate), all three ligands caused significant amounts of precipitation (Fig. 2). The ligand concentration where the precipitation curve leveled off was directly used for subsequent capturing (precipitation) of Gal-3 in bulk cell lysate. The abundance of Gal-3 in different batches was normalized based on hemagglutination titer values of the cell lysates. Through this approach, the ratios between the capturing agents and the lysates from different batches were properly maintained. Tg, CSA, and CSC turned out to be efficient capturing agents for Gal-3 (Fig. 2).

Release and separation of captured Gal-3 from the TCAs—Galectin-3–TCA complexes were isolated by centrifugation at 6339 × g at 4 °C for 30 min. The complexes were dissolved by 300 mM β-lactose solution in PBS to release captured Gal-3. The solution containing the dissolved complexes was placed in an Amicon separation/filtration unit (the pore size of the separating membrane was 50 μm). The molecular mass of a Gal-3 monomer is ~29 kDa; therefore, it passed through the filter and accumulated in the bottom compartment of the unit, whereas Tg (molecular mass, ~670 kDa) stayed on the top of the filter (upper compartment of the unit). After exhaustive filtration, the Tg from the upper compartment could be recycled as the lectin capturing agent. The separated Gal-3 was dialyzed against PBS to remove β-lactose. The purity of CaRe-purified Gal-3 was checked by SDS-PAGE, which showed a single band at 29 kDa (Fig. 2). Gal-3 captured by CSA and CSC and released by β-lactose showed similar bands (Fig. 2). The efficacy of CSA and CSC as capturing agents of Gal-3 is consistent with our previous observation that CSA and CSC are multivalent ligands of Gal-3 (31).

**Purification of ConA by CaRe**

Invertase and mannann were used as capturing agents for ConA—Crude extract from Jack bean (Canavalia ensiformis) flour was prepared with 100 mM HEPES buffer (pH 7.2) containing 150 mM NaCl, 5 mM CaCl₂, and 5 mM MnCl₂. The crude preparation showed hemagglutination activity when tested with rabbit erythrocytes. Among the multivalent ligands tested, invertease and mannann inhibited the hemagglutination activity of the crude extract. Invertease is a mannglycoprotein that contains several accessible high-mannose glycan chains (34) and, thus, a multivalent ligand of ConA. When tested for their ability of capture (precipitate) ConA in the crude extract, both invertase and mannann showed significant cross-linking activity toward ConA (Fig. 3, A and B). Thus, ConA was captured in the crude extract by using invertase and mannann as TCAs.

**Release and separation of captured ConA from the TCAs**—Captured ConA was released when ConA–TCA complexes were incubated with 300 mM α-methyl mannose in 10 mM sodium acetate buffer containing 5 mM CaCl₂, 5 mM MnCl₂, and 150 mM NaCl (pH 5.2). The subunit molecular mass of ConA is 26 kDa. At pH 5.2, the lectin exists as a dimer (molecular mass, 52 kDa) (35). Dimeric molecular mass ensured proper separation of ConA from its relatively higher-molecular-mass TCAs. SDS-PAGE profiles of the separated ConA demonstrate that the CaRe procedure efficiently purified ConA from the crude extract prepared from Jack bean meal (Fig. 3). SDS-PAGE profiles of purified ConA (including commercially available purified ConA) showed additional low-molecular-mass bands along with the ~26-kDa subunit (Fig. 3E). These low-molecular-mass bands were not contamination or impurities; they were part of the pure lectin generated from the 26-kDa subunit by proteolytic degradation. This is an inherent property of purified ConA.

SBA is a tetrameric lectin that is also a glycoprotein. SBA possesses four high-mannose (Man₉) glycan chains per tetramer (36). It can bind to complex-type glycans via its glycan-binding sites, and it can also bind to mannose-binding lectins via its high-mannose glycan chains. Thus, SBA can serve as a bifunctional tool. In this study, it served as a lectin (captured by a complex-type glycoprotein, asialofetuin (ASF)), as a capturing agent (for capturing ConA), and as a glycoprotein (that was captured by a lectin). SBA is a multivalent ligand of ConA (35). When used as a capturing agent of ConA, SBA was able to efficiently capture ConA in the crude extract (Fig. 3C). Upon releasing the captured ConA by α-methyl mannose, ConA was...
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Figure 2. Purification of recombinant human Gal-3 by CaRe. A–C, Gal-3 in E. coli lysate was captured with bovine Tg (A), CSA (B), and CSC (C). The graphs show concentration-dependent cross-linked complex formation by Tg, CSA, and CSC, respectively. These profiles also indicate the capturing abilities of different TCAs. OD, optical density. D, SDS-PAGE profiles of molecular mass markers (lane 1), Gal-3 purified with Tg (lane 2), CSA (lane 3), and CSC (lane 4) as capturing agents.

Separated from SBA by 100-kDa membrane filtering at pH 5.2. As the molecular mass of ConA at this pH is 52 kDa, it passed through the filter, whereas SBA (molecular mass, 120 kDa) stayed at the top of the filter. The SDS-PAGE profile of this separated ConA was similar to those of other purified ConA samples (Fig. 3E, lane 5).

Purification of SBA by CaRe

Soybean agglutinin is a GalNAc/galactose/LacNAc–specific tetrameric lectin with a native molecular mass of 120 kDa (four subunits of 30 kDa each). As mentioned above, the glycan-binding sites of SBA interact with complex-type glycan containing glycoproteins such as ASF (37).

ASF was able to capture SBA in the crude extract of soy flour. Invertase was an efficient capturing agent for ASA—Invertase, mannan, and Tg were tested for their ability to capture ASA in the crude extract. Invertase was selected as the capturing agent for ASA because it showed a greater ASA-capturing (precipitation) ability than mannan and Tg (Fig. 4, A–C).
Release and separation of ASA—The invertase–ASA complex was dissolved with 300 mM α-methyl mannose in PBS to release the captured ASA. The native molecular masses of ASA and invertase were ~25 kDa (two subunits of 12.5 kDa) and 270 kDa, respectively. An Amicon unit with a 100-kDa filter separated ASA from invertase. After filtration, ASA accumulated at...
the lower chamber of the unit, leaving invertase at the top chamber. The SDS-PAGE profile of the sample at the lower chamber showed a single band of ~12.5 kDa (Fig. 4D).

**Purification of lectins from C. esculenta and X. sagittifolium by CaRe**

*C. esculenta* lectin and *X. sagittifolium* lectin belong to a large superfamily of mannose-binding proteins (39). These two lectins bind to high-mannose and complex-type N-glycans. Therefore, we decided to use high-mannose– and complex-type N-glycan– containing glycoproteins as capturing agents to purify these two lectins.

Invertase, bovine Tg, and ASF were employed as capturing agents—Based on the inhibition properties, invertase and Tg were tested for their ability to capture *C. esculenta* lectin. As indicated by the precipitation profiles (Fig. 5, A and B), invertase and Tg strongly interacted with *C. esculenta* lectin in the crude extract and formed substantial amounts of cross-linked precipitates. Therefore, we used these two glycoproteins as capturing agents to purify *C. esculenta* lectin. For *X. sagittifolium* lectin, we tested invertase and ASF for their capturing potential. Compared with ASF, invertase showed stronger cross-linking properties with *X. sagittifolium* lectin (Fig. 5, C and D). Thus, we used invertase as the capturing agent for *X. sagittifolium* lectin.

**Release and separation of C. esculenta lectin and X. sagittifolium lectin from their TCAs—**C. esculenta lectin and *X. sagittifolium* lectin were released by lowering the pH of the solution. Sodium acetate buffer (pH 4.9) was used for this purpose. The released lectins were separated by membrane filtration. On SDS-PAGE, the CaRe-purified lectins showed char-
acteristic bands without any impurities (Fig. 5E). We decided to use a buffer with a relatively low pH (4.9) for dissociation of lectin–TCA complexes based on the experience we gained in a similar situation. When precipitates (formed by lectin–ligand cross-linking interactions) are difficult to dissolve by competitive mono- or disaccharides, the easiest way to dissolve such complexes is to lower the pH. When the pH (4.9) of the samples was increased to 7.4 by dialyzing them against PBS (pH 7.4), both lectins were able to agglutinate rabbit RBCs. Thus, exposure to sodium acetate buffer (pH 4.9) did not impair the lectins.

**Application of CaRe for glycoprotein purification**

Besides lectins, CaRe was also effective for glycoprotein purification. As a proof of concept, the plant glycoprotein SBA was purified by CaRe. As mentioned above, SBA is a mannosglycoprotein that possesses one high-mannose (Man₉) glycan chain per subunit (four glycan chains per tetramer of SBA). Therefore, it is a high-affinity multivalent ligand of mannose-specific lectin ConA (35). Taking advantage of this property, ConA was used as a capturing agent to purify SBA from crude extract (Fig. 6A). Captured SBA in the precipitated complex was released by 300 mM α-methyl mannose solution in 100 mM sodium acetate buffer (pH 5.2). Both ConA and SBA are tetrameric proteins with almost identical native molecular masses at pH 7.2. Thus, separation of SBA from its capturing agent ConA was challenging. As mentioned above, the tetrameric ConA becomes a dimeric protein at pH 5.2, with a native molecular mass of 52 kDa (35). In contrast, SBA exists as a tetramer at pH 5.2, with a native molecular mass of 120 kDa (35). Thus, SBA was separated from its capturing agent ConA by size exclusion chromatography at pH 5.2 (Fig. 6C). SBA separated this way was pure, as it showed a single band of 30 kDa on SDS-PAGE (Fig. 6D). When separation was attempted by membrane filtration, most
ConA passed through the 100-kDa filter, but traces of ConA still remained mixed with SBA on the filter in the upper chamber. This problem was avoided by separating SBA from ConA by gel filtration, and thus SBA was found in pure form (Fig. 6D).

Interestingly, besides pure ConA, crude ConA (in Jack bean flour extract) was also able to capture SBA in the crude soy flour extract when mixed in proper ratios. In other words, SBA was captured and purified even when the capturing agent (ConA) was not pure. Substantial capturing of SBA occurred when the two crude extracts were mixed in a concentration-dependent manner (Fig. 6B). Subsequent release and separation of SBA were performed as described above. SBA separated this way showed a single band of 30 kDa on SDS-PAGE (Fig. 6D).

The ability of CaRe to purify glycoproteins was further demonstrated by isolating two other glycoproteins: C. esculenta lectin (Tarin) and chicken ovalbumin. Like SBA, C. esculenta lectin is a glycosylated protein (39), and ConA can bind to its covalently linked glycan chains. Ovalbumin, a constituent of chicken egg white, contains oligomannose and/or hybrid-type oligosaccharides, which are binding epitopes for ConA (35).

Thus, ConA was used as the capturing agent for both glycoproteins (Figs. 7 and 8). After capturing by ConA, the glycoproteins were released by 300 mM α-methyl mannose solution. The glycoproteins were separated from ConA by using 50-kDa and 30-kDa filters. The separated glycoproteins showed single bands on SDS-PAGE (Figs. 7 and 8).

As demonstrated above, when a lectin itself is a glycoprotein, it can be purified by CaRe following two different strategies: it can be captured by a glycoprotein that specifically binds to the glycan binding site of the target lectin, and the target lectin can

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**Figure 6. Purification of glycoprotein SBA by CaRe.** A and B, SBA in crude soybean meal was captured by pure ConA (A) and ConA in crude Jack bean extract (B). OD, optical density. C, captured SBA was released from its capturing agent (ConA) by α-methyl mannose and separated from its capturing agent (ConA) by gel filtration on a Biogel P-100 column. D, SDS-PAGE profiles of molecular mass markers (lane 1), commercially obtained purified SBA (lane 2), and SBA purified by capturing with ConA (lanes 3 and 4).
also be captured by another lectin that specifically interacts with the covalently linked glycan moieties of the target lectin. Experiments with glycoproteins demonstrate that lectins can be used as capturing agents with the CaRe method to purify a variety of glycoproteins and possibly other glycoconjugates, including proteoglycans.

Discussion

When the valency of a lectin is two or more, it forms cross-linked complexes with multivalent glycoconjugates (30, 40). CaRe has been developed based on this intrinsic property of lectin–glycoconjugate interaction. In this study, we have shown that CaRe can efficiently purify new and known lectins and that the principles of CaRe can also be used for purifying glycoproteins. The percent yield of the lectins by CaRe was comparable with that of traditional affinity chromatography. Multivalent glycoproteins can serve as lectin-capturing agents, whereas multivalent lectins can be employed as glycoprotein-capturing agents (Table 1). In principle, multivalent lectins as capturing agents can isolate other glycoconjugates, such as proteoglycans and glycolipids, as long as they have a functional valency of two or more for the target glycoconjugates. The ability of human Gal-3 to interact with glycoproteins, glycosaminoglycans, and proteoglycans as described in this study and elsewhere (31) makes Gal-3 a bifunctional capturing agent able to purify both glycoproteins and proteoglycans. We are currently testing this possibility. CaRe can also be useful for isolating nonlectin proteins when they are multivalent for their specific capturing agents. Thus, CaRe can serve as an effective tool for lectinomics, glycoproteomics, and proteomics.

The success of CaRe depends on proper selection of the capturing agents. Capturing agents should have the ability to bind and form insoluble cross-linked complexes with the targets, and their molecular masses should not be the same or close to those of their respective targets. The ability of multivalent glycan-binding proteins to form insoluble cross-linked complexes with multivalent glycoconjugates is an inherent feature. Nevertheless, the cross-linking ability of each multivalent ligand must be tested before it can be considered as a TCA. In this study, we selected TCAs (glycoproteins and polysaccharides) based on
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the specificities of the target lectins. However, when the targets and their glycan specificities are unknown, TCAs can be selected using three simple steps, as described below.

All lectins and glycoproteins purified by CaRe in this study are known proteins. Although most of the glycoprotein binding properties and the capturing abilities of the glycoproteins and polysaccharides were elucidated in this study, information regarding the molecular mass and glycan specificities of the proteins was available in the literature. Such known proteins were deliberately chosen for proper validation of this new technique. The known characteristics of these proteins helped establish the reliability of CaRe. However, this protocol should be equally effective for detecting and purifying completely unknown lectins and glycoproteins. This could be accomplished without any prior information regarding the target lectins/glycoproteins. Information necessary for performing CaRe (such as selection of TCAs) can be obtained by three simple and quick steps (described under “Experimental procedures”). The first step is measuring the lectin activity of crude preparations (extracts or cell lysates) by hemagglutination test. The second step is determining the specificity of the unknown targets. If a crude extract under investigation shows agglutination, then an inhibition test with strategically selected ligands can easily determine the specificity of the target lectin present in the crude extract. The panel of ligands should contain mono- and disaccharides as well as glycoproteins. Inhibition data with mono- and disaccharides (for example, galactose, GalNAc, mannose, lactose, LacNAc, sialic acid, and fucose) indicate the specific group to which the target lectin belongs (whether the target lectin is galactose/GalNAc-specific, mannose-specific, sialic acid-specific, or fucose-specific). These assays may also show whether a crude extract contains more than one lectin. When the mono- or disaccharide specificity of the target lectin has been determined, its binding interactions with glycoproteins are tested. This is important because one or more of these glycoconjugates would be tested for their suitability as TCAs. It should be noted that some lectins do not show any inhibition profile with mono- and disaccharides. The specificity of these lectins should be elucidated by using glycoproteins. The third step is finding TCAs. When the specific glycoprotein ligands of the target lectin are identified, the glycoproteins are tested for their ability to form noncovalent cross-linked complexes with the target lectin. This is done by precipitation assays. The most efficient cross-linker glycoconjugates are selected as TCAs. Based on our studies, the following glycoconjugates could be suitable TCAs for specific lectin groups: invertase, SBA, and mannan for mannose-specific lectins; ASF for galactose/LacNAc/GalNAc-binding lectins that are unable to bind terminal sialic acid residues of complex-type glycans; and fetuin and Tg for galactose/LacNAc/GalNAc-binding lectins that can tolerate/bind terminal sialic acid residues of complex-type glycans. The list of glycoproteins could be extended based on the binding properties of the target lectins. Extensive trial and error could be avoided by performing these three quick and easy steps, which can be completed in 4–6 h. In other words, it is possible to extract relevant information (necessary for CaRe) regarding completely unknown targets in a single day.

When the targets are unknown glycoproteins, only the third step can be used. To identify and purify unknown glycoproteins, lectins can be used as TCAs. Plant lectins can serve as efficient TCAs because of their diverse glycan specificities, easy availability, and oligomeric structures (important for cross-linked complex formation). A panel of plant lectins with different specificities should be tested for their capturing (complex-forming/precipitating) abilities of unknown glycoproteins in crude samples. The lectin(s) with the highest capturing abilities should be selected as TCAs of glycoproteins. Plant lectins would be ideal TCAs for all kinds of glycoproteins (high-mannose, sialylated complex-type, asialo complex-type, hybrid-type, and mucin-type).

Unknown lectins and glycoproteins separated by CaRe, as described above, can be identified by proteomic analysis. If a TCA captures more than one target, they can be separated by SDS-PAGE, and then individual bands can be analyzed and identified by mass spectrometry analysis. Thus, target lectins and glycoproteins having no prior information can be purified by CaRe by performing the three simple steps described above (descriptions of these three steps are given under “Experimental procedures”). Therefore, CaRe could potentially be a valuable research tool for discovering completely unknown lectins and glycoproteins.

Each plant lectin purified here by CaRe was one of the most abundant proteins (and possibly the only lectin) in the respective plant crude preparation. Recombinant human Gal-3 purified by CaRe was overexpressed in E. coli. Questions remain regarding the usefulness of CaRe in situations where a crude preparation contains more than one lectin and the target lectin is at normal physiological concentration (such as human Gal-3 in human cells or tissues). If a crude extract contains multiple lectins, then they can be identified using the simple three steps described above, and then they can be purified individually by CaRe. Tissue and cell extracts (containing lectins and glycoproteins at physiological concentrations) could be concentrated by membrane filtration before performing CaRe. Such filtration would increase the concentration of target lectin/glycoprotein to a level detectable by CaRe. The small volume of the samples derived from human cell or tissue sources would not be an impediment for CaRe because this method is capable of identifying/purifying targets from a few milliliters (as low as ~1 ml) of crude preparations. Our ongoing work indicates that CaRe would be an effective tool for detection/purification of targets (lectins and glycoproteins) from human/mammalian samples.

CaRe could potentially be employed to identify and purify endogenous binding partners of lectins and glycoconjugates. For example, if Gal-3 is used as a capturing agent in a cell or tissue extract, then the lectin can potentially capture and isolate some of its endogenous ligands. Similarly, glycoconjugates of microbial origin and tailored synthetic glycans with pathologically significant epitopes as capturing agents can help identify putative natural binding partners with immunological functions.

If target lectins or glycoconjugates function as a part of a protein complex, then CaRe can potentially isolate that complex along with the targets. In this study, however, no other proteins were copurified along with the targets. This observa-
tion indicates that lectins purified in this study were possibly not part of any protein complexes.

We wanted to compare CaRe with immunoprecipitation (IP) and co-IP, also known as a pulldown assay, because they also capture target proteins in solution phase and use smaller sample volumes. Although carbohydrate–protein interaction is the basis of finding and capturing the targets in CaRe, targets are pulled down via protein–protein interactions in immunoprecipitation. IP and co-IP are extensively used methods (41–46) that often involve elaborate arrangements and require antibodies against the target proteins (47). Therefore, it is crucial for these methods that at least one target protein should be known (48). In addition, the success of IP and co-IP depends on multiple experimental conditions (48). In contrast, CaRe is straightforward, and this method can purify both known and completely unknown targets because it does not require antibodies against the targets.

Antibodies, the capturing agents in IP and co-IP, cannot work effectively unless they are attached directly to agarose beads or superparamagnetic microbeads or to protein A/G attached to the beads (49–51). However, the antibodies, the beads, and protein A/G can nonspecifically interact with other biomolecules present in the crude lysate. Coisolation of entities other than the target protein(s) can seriously jeopardize the purpose of IP and co-IP. The chance of nonspecific binding in CaRe is minimal, as target lectins are captured via specific carbohydrate–protein interactions, and this procedure does not use antibodies, beads, or protein A/G. If the crude extract contains more than one target lectin with a similar binding specificity, then they can be copurified with the same TCA, but the chance of nonspecific interactions with other biomolecules is very low. Selection of the proper bead type and the amount of antibodies on the beads may influence the outcome of IP and co-IP. Such issues are nonexistent in CaRe because beads are not used in this technique. In addition to nonspecific binding, antibody contamination is a major problem encountered in both IP and co-IP (52). Target proteins are often coeluted with capturing antibodies, especially when the elution is done under denaturing conditions. CaRe does not use antibodies, and target proteins are never separated from the TCAs under denaturing conditions. Therefore, the problem of contamination is absent in CaRe. In co-IP, fusion tags are often inserted into the target proteins so they can be captured by anti-fusion tag antibodies. CaRe does not require fusion tags to capture unknown targets.

The principles of CaRe and the conventional immunoprecipitation method could be combined to develop a powerful separation technique. In this approach, beads could be coated with glycoconjugates instead of antibodies to capture lectins, or the beads could be coated with lectins to capture glycoconjugates. Clustering of multivalent capturing agents on beads will further enhance their target-capturing ability. This approach would particularly be useful for monovalent targets. We are currently examining the feasibility of this approach. CaRe is a relatively rapid, simple, and affordable method that can purify targets from smaller amounts of initial samples. This method does not involve steps of functionalization or covalent modifications. Given all of the advantages, CaRe could be used as an effective tool for purifying and identifying new lectins, glyco-
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centrifuged at 1957 × g for 30 min at 4 °C. The supernatant was recentrifuged at 9469 × g for 30 min at 4 °C. The supernatant was subjected to two-step ammonium sulfate precipitation (30% and 80%) as described above for soy flour crude extract. The precipitates from the 80% ammonium—treated crude extract was collected and divided into two portions. One portion was diazylated against HEPES (pH 7.2) buffer, and the other portion was diazylated against 100 mM sodium acetate buffer (with 150 mM NaCl, 5 mM Calci2+, and 5 mM Mncl2 (pH 5.2)). Jack bean extracts prepared in two different buffers were selectively used for CaRe based on specific experimental requirements.

Corms of *C. esculenta* (200 g) were homogenized in 500 ml of PBS (pH 7.4). After stirring overnight at 4 °C, the mixture was centrifuged at 1957 × g for 30 min at 4 °C. The supernatant was centrifuged again at 9469 × g for 30 min at 4 °C. The supernatant was used for CaRe. Crude extracts from the corms of *X. sagittifolium* and *A. sativum* bulbs were prepared following a similar procedure and using similar tissues/buffer ratios. Crude extracts prepared as described above, except the buffers used were either 100 mM HEPES (pH 7.2) or 100 mM sodium acetate (pH 5.2). These two buffers contained 5 mM Calci2+ and 5 mM Mncl2. This was done to make sure that ConA, the TCA of these glycoproteins, was fully active. ConA needs calcium and manganese ions for proper activity. Extracts of soybean flour and *C. esculenta* corms thus prepared were used for purification of glycoprotein SBA and *C. esculenta* lectin (Tarin), respectively.

Egg whites from nine chicken eggs were collected after discarding the yolks. The egg whites were placed in a blender containing 50 ml of 100 mM HEPES buffer (pH 7.2). The egg whites in HEPES were homogenized for 90 s (three times for 30 s each), and the solution was left to stir for 2 h at 4 °C. The mixture was centrifuged at 9469 × g for 30 min at 4 °C. The supernatant was used for purification of ovalbumin by CaRe.

**Detection of lectin activity in the crude extract**

Lectin activities of the clarified crude extracts were detected by hemagglutination assay using rabbit red blood cells (RBCs). Freshly drawn rabbit blood (3 ml) was added to 10 ml of 20 mM PBS (or other buffers based on the experimental requirements) and centrifuged at 313 × g for 10 min at 4 °C. The supernatant was discarded. RBCs accumulated at the bottom of the centrifuge tubes were suspended in 10 ml of the same buffer and centrifuged again under similar conditions. The process was repeated four more times. The washed packed RBCs were used to make a 2% (v/v) RBC suspension in buffer that was used for hemagglutination assays and inhibition tests. Hemagglutination assays were performed using 96-well U-bottom microtiter plates. An equal volume of buffer (25 μl) was first added to each well, and then 25 μl of a crude sample was serially diluted from 1% to 0.001% (in 10-fold serial dilution technique). After incubation, the mixtures were centrifuged at 6339 × g for 30 min to separate the precipitate (TCA—lectin complex) from other components. The precipitate contained the target lectins and was washed three times with buffer (the temperature of the buffer was lowered by storing it at 4 °C before use) by centrifugation at 6339 × g at 4 °C (Fig. 1).

**Inhibition assays to identify multivalent inhibitors as TCAs**

The inhibition assays were performed at room temperature using 96-well U-bottom microtiter plates and a 2-fold serial dilution technique (31) with 2% (v/v) rabbit erythrocytes in 20 mM PBS containing 150 mM NaCl or in HEPES buffer. Each ligand (25 μl) was serially diluted in wells containing 25 μl of buffer in each well. After incubation, 25 μl of crude extract was added to each well, mixed gently, and incubated for 60 min. After incubation, 25 μl of rabbit RBCs (2% (v/v)) was added to each well. The titer plate was shaken gently and then kept undisturbed. Results were noted after 1 h. The minimum concentration of ligands required for complete inhibition of three hemagglutination doses was determined.

**Determining the capturing potential of TCAs by precipitation experiments.**

Multivalent ligands that inhibited hemagglutination activities of the crude extracts containing the target lectins were subjected to precipitation (noncovalent cross-linking) experiments. This was done to determine their capturing abilities. In this assay, increasing amounts of TCAs (multivalent ligands) were added to a series of tubes containing equal amounts of crude samples. The precipitation was allowed to occur at room temperature for 1–12 h depending on the samples. The amount of precipitation was measured at 420 nm with a Shimadzu UV-2450 spectrophotometer. The precipitation curves were used to compare the relative capturing efficiency of the TCAs for the target lectins and to determine the binding stoichiometry (ratio of TCA and crude) required for obtaining maximum precipitation (capture).

**Capturing of lectins in the crude extracts by TCAs and separation of the TCA–lectin complex from other components of the crude extracts**

After the most potent TCAs for each target lectin were identified, the TCAs were mixed with their corresponding crude extracts at proper stoichiometric ratios to capture the maximum amount of target lectins in the cross-linked precipitates. TCA/crude mixtures were incubated at 4 °C for 1–12 h. After incubation, the mixtures were centrifuged at 6339 × g at 4 °C for 30 min to separate the precipitate (TCA—lectin complex) from the supernatant. The supernatant containing other components was discarded. Precipitates containing the TCA and the target lectins were washed three times with buffer (the temperature of the buffer was lowered by storing it at 4 °C before use) by centrifugation at 6339 × g at 4 °C (Fig. 1).

**Releasing captured lectins by dissociating the TCA–lectin complex**

To release the target lectins from the TCAs, the precipitates (TCA–lectin complexes) were dissolved by incubation for 4 to
18 h with specific monovalent saccharide ligands in PBS (pH 7.4) (Fig. 1). In certain cases, monovalent saccharide ligands were unable to dissolve the TCA–lectin complexes. In those situations, the captured lectin was released by treating the complexes with a low-pH buffer (such as 100 mM sodium acetate buffer (pH 4.9)). Our previous experience (where we found that, when competitive ligands failed to dissociate lectin–glycoprotein complexes, dissociation could be accomplished by lowering the pH) helped us with this strategy. When the pH was increased to 7.4 after dissociation and separation, the lectins were fully active. Thus, temporary exposure to a lower pH (pH 4.9) did not damage the lectins.

Separation of lectin from TCA

The released target lectin and TCA were separated by membrane filtration or size exclusion chromatography (Fig. 1). For filtration, Amicon membrane filtration tubes with an appropriate molecular mass cutoff (100 kDa, 50 kDa, and 30 kDa) were used. For size exclusion chromatography, a 177 × 1.5 cm column packed with Biogel P-100 beads was used. The column was prepared and calibrated according to the instructions provided by Bio-Rad. Protein fractions were collected in ~1.5-ml portions, and the absorbance was detected at 280 nm with a Shimadzu UV-2450 spectrophotometer. After separation from TCA, the purity of lectins was checked by SDS-PAGE using a Bio-Rad Mini–PROTEAN Tetra Cell following the manufacturer’s instructions. Precast gels were purchased from Bio-Rad. The gel was stained using Coomassie Brilliant Blue.

Purification of glycoproteins by CaRe

Besides lectins, the CaRe method was employed to purify glycoproteins from crude extracts. As a proof of concept, the glycoprotein SBA was purified by using ConA as a capturing agent. In this approach, all steps were similar to those described above for CaRe, except a lectin (ConA) was used as the capturing agent for the glycoprotein (SBA). Captured SBA was released by 300 mM α-methyl mannose and separated from ConA by gel filtration on a Biogel P-100 column at pH 5.2 (100 mM sodium acetate buffer, 150 mM NaCl, 5 mM CaCl₂, and 5 mM MnCl₂). CaRe was employed to purify two more glycoproteins, C. esculenta lectin or Tarin and ovalbumin from egg whites. In both cases, ConA was used as the capturing agent. Captured ovalbumin and Tarin were released from ConA by 300 mM α-methyl mannose and separated from ConA by membrane filtration. After separation, the purity of the glycoproteins was checked by SDS-PAGE.

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