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NOTE

An alternative easy method for antibody purification and analysis of protein–protein interaction using GST fusion proteins immobilized onto glutathione–agarose

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Abstract Immobilization of small proteins designed to perform protein–protein assays can be a difficult task. Often, the modification of reactive residues necessary for the interaction between the immobilized protein and the matrix compromises the interaction between the protein and its target. In these cases, glutathione-S-transferase (GST) is a valuable tag providing a long arm that makes the bait protein accessible to the mobile flow phase of the chromatography. In the present report, we used a GST fusion version of the 8-kDa protein serine protease inhibitor Kazal-type 3 (SPINK3) as the bait to purify anti-SPINK3 antibodies from a rabbit crude serum. The protocol for immobilization of GST-SPINK3 to glutathione–agarose beads was modified from previously reported protocols by using an alternative bifunctional cross-linker (dithiobis(succinimidyl propionate)) in a very simple procedure and by using simple buffers under physiological conditions. We concluded that the immobilized protein remained bound to the column after elution with low pH, allowing the reuse of the column for alternative uses, such as screening for other protein–protein interactions using SPINK3 as the bait.

Keywords Antibody purification · Protein–protein interactions · Glutathione-agarose column · DSP cross-linker · SPINK3

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When recombinant proteins are expressed as glutathione-S-transferase (GST) fusions, they are usually subsequently cleaved by thrombin, protease X, or TEV protease. However, one can take advantage of the GST fusion construct since the bait protein can be affinity-retained onto glutathione–agarose beads, and then, GST can be easily cross-linked to glutathione–agarose beads. This could be beneficial for most small proteins or proteins that would not be exposed if directly cross-linked to a CNBr-activated resin. GST protein provides a long arm, making possible the exposure of the bait protein to the mobile phase of the chromatography. Thus, it allows the interaction between the immobilized protein and a mixture of proteins included in a fluid or protein extract. The immobilization of GST fusion proteins to glutathione–agarose beads has been historically performed by using the cross-linker dimethyl pimelimidate–HCl (DMP)^2 [1]. DMP requires a working solution of high pH (0.2 M borate–NaOH, pH 8.6) that is not compatible with the preservation of native structure of some proteins, which can be a problem when protein–protein interaction is to be evaluated. In this work, we report an alternative method using the bifunctional cross-linker dithiobis(succinimidyl propionate) (DSP), an amine-reactive cross-linker having *N*-hydroxysuccinimide (NHS) ester reactive ends and a cleavable disulfide bond in its spacer arm. DSP contains an amine-reactive NHS ester at each end of an eight-carbon spacer arm. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for NHS ester cross-linking reagents. Moreover, DSP can be used with a more physiological buffer compared to DMP, the phosphate saline buffer (pH 7.4).

To evaluate the efficiency and reliability of the method, we evaluated an application of this method by purifying specific immunoglobulins against serine protease inhibitor Kazal-type 3 (SPINK3) from crude rabbit sera. Polyclonal antibodies against

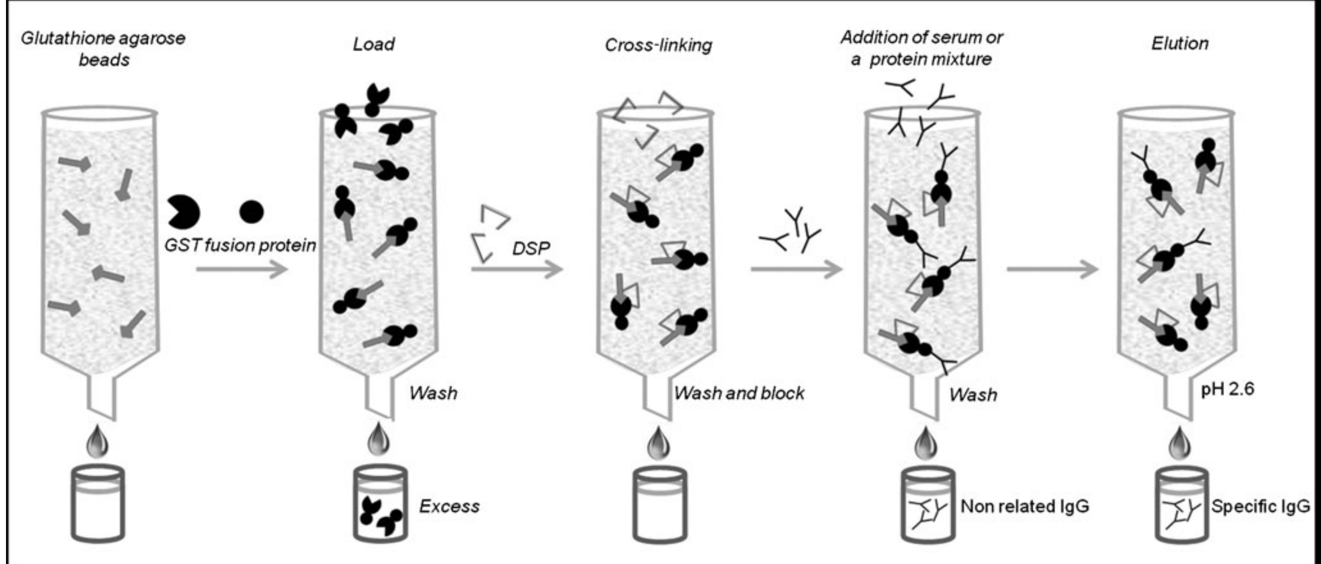


Fig. 1 Schematic representation of a method for antibody purification or detection of ligands' binding affinity to a GST fusion protein. A glutathione (GSH)–agarose affinity column was equilibrated and the GST fusion protein (GST portion symbolized as ◖ + bait protein symbolized as ●) was loaded onto the matrix. The column was washed to remove the excess of fusion protein, and thereafter, GST fusion protein and GSH were cross-linked by 2 mM DSP at 4 °C. The reactive groups were blocked and the uncross-linked protein was washed with glycine–HCl pH 2.6 and subsequently neutralized. Once the GST fusion protein was already immobilized and the column equilibrated, an aliquot of crude antiserum (or a protein mixture) was loaded and incubated onto the column. Before elution with 0.2 M glycine–HCl pH 2.6, the non-related IgG fraction was washed. The eluted fraction was collected in tubes containing 2 M Tris–HCl pH 9 in order to neutralize the antibody solution. Finally, the column was regenerated, equilibrated, and stored at 4 °C until the next use

recombinant proteins usually are generated in rabbit or mouse serum which is subsequently pre-adsorbed with the host extract (i.e., *Escherichia coli* strains) to eliminate cross-reaction with unspecific proteins that frequently contaminate the desired protein. Nevertheless, it usually gives background bands in Western blots or false positives in immunocytochemistry.

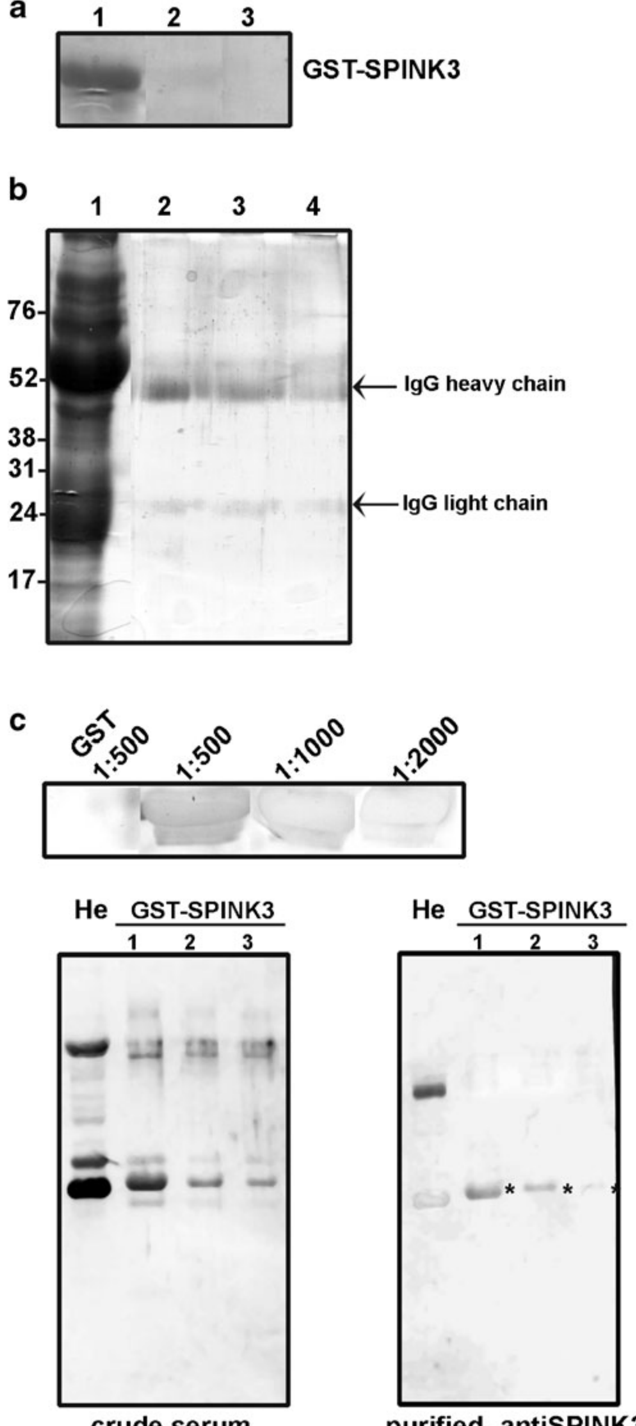
In this method, once the matrix is prepared by employing a three-step simple protocol that takes 2 h, the crude serum is loaded onto a column. After a 15-min incubation period, the unbound proteins are eliminated and the retained specific antibody is further eluted by lowering the pH of the buffer solution (Fig. 1).

Recombinant protein purification and construction of the affinity beads

The cDNA encoding SPINK3 from *Mus musculus* (NCBI ID: NM_009258.5) was cloned into a pGEX-4T-3 expression vector (GE) downstream of the GST coding sequence as we described previously [2]. The overexpression of GST-SPINK3 in *E. coli* Rosetta cells (Novagen) was performed as described [2] starting from a 400-ml culture. The soluble fraction (20 ml) was loaded onto a Glutathione Sepharose 4B affinity column (1 ml) equilibrated with phosphate buffer saline (PBS), and the excess of protein was washed with the same buffer. Cross-linking of GST-SPINK3 to glutathione–agarose was initiated by addition of 2 mM DSP, and the mixture was incubated for 1 h at 4 °C under gentle agitation. DSP-reactive groups were then blocked by incubation in 1 M Tris–HCl pH 7.5 for 15 min at room temperature, and the column was re-equilibrated with PBS. To remove uncross-linked protein, the column was washed with two volumes of 0.2 M glycine–HCl pH 2.6 and neutralized with two volumes of 0.1 M $NaHCO_3$ containing 0.5 M NaCl and the column was finally equilibrated with PBS. The reliability of the cross-linking procedure was confirmed since no leakage of proteins was observed after the first wash with the elution buffer. All the steps of the column preparation were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brillant blue staining (Fig. 2A).

Antibody purification

Once the GST-SPINK3 was immobilized in the agarose matrix, 3 ml of crude rabbit serum containing polyclonal anti-SPINK3 antibodies raised against a His-tagged version of the protein previously produced (SPINK3-His_6) [3] was diluted 1:1 in PBS and loaded onto the column. Interaction was



◀ **Fig. 2** GST-SPINK3 immobilization to glutathione–agarose beads and antibody purification. (**A**) Monitoring the column by SDS-PAGE (12 %). The soluble fraction of an *E. coli* (Rosetta) protein extract that overexpressed GST-SPINK3 was loaded onto a Glutathione Sepharose 4B affinity column (*lane 1*), and cross-linking was initiated by addition of 2 mM DSP. Uncross-linked protein was washed with 0.2 M glycine–HCl pH 2.6 (*lane 2*). After re-equilibration, the reliability of the cross-linking procedure was confirmed by analyzing the leakage produced by the elution buffer (*lane 3*). (**B**) Purification of anti-SPINK3. *Lane 1*: crude anti-serum; *lanes 2–4*: consecutive elution fractions containing the purified antibody. The position of the molecular mass markers is indicated on the left. (**C**) Titration and evaluation of cross-reactivity of the antibody. The *upper panel* represents the antibody titer analyzed by Western blot. It shows positive reactivity of three serial dilutions of the purified antibody (1:500, 1:1,000, 1:2,000) against GST-SPINK3 (2.5 μg) and the negative cross-reactivity against GST (2.5 μg). The *lower panel* shows the Western blot assay performed with either crude antiserum (1:1,000) or purified anti-SPINK3 (1:1,000). *Lane He*: 20 μg protein from heart extract; *lanes GST-SPINK3* (1–3): 3, 1, and 0.5 μg of purified GST-SPINK3. Possition of GST-SPINK3 band is indicated by *

facilitated by overnight (ON) incubation at 4 °C under gentle agitation. After washing the unbound protein fraction with ten volumes of PBS, anti-SPINK3 antibodies were eluted with three volumes of 0.2 M glycine–HCl pH 2.6. Fractions (0.5 ml) were collected on tubes containing 20 μl of 2 M Tris–HCl pH 9 in order to neutralize the antibody solution. Purified anti-SPINK3 was dialyzed against PBS/glycerol (1:1); then, 1 % (*w*/*v*) BSA was added and aliquots of the antibody were stored at −80 °C.

The column was regenerated with four volumes of 1 M Tris–HCl pH 8, re-equilibrated with PBS, and stored at 4 °C with 0.02 % sodium azide. The yield of the specific antibody as evaluated by SDS-PAGE was about 500 μg in a concentration of 0.33 mg/ml (Fig. 2B).

Titration and reactivity of the antibody

The titer of the purified antibody solution was determined by Western blot against pure GST-SPINK3 as 1:2,000. GST was used as a control of unspecific interactions (Fig. 2C, upper panel). To determine the sensitivity and cross-reactivity of the antibody against unspecific proteins, non-purified serum and purified serum were compared by Western blotting on three different amounts of purified GST-SPINK3 and a mouse heart protein extract. The purified antibody anti-SPINK3 effectively recognized up to 0.5 μg of the recombinant protein (Fig. 2C, lower panel, lanes 1–3), and its specificity was improved as can be observed by the decrease in the number of cross-reactive bands in the heart protein extract, similar to that shown by commercial antibodies against SPINK3 on the same tissue (data not shown).

We have described a fast and efficient protocol to immobilize a GST-tagged protein to glutathione–agarose beads by using an alternative cross-linking reagent and by using simple reagents. An important advantage is that the column can be reused several times. This claim is based on the evidence that no leakage of the fusion protein was observed (Fig. 2A, lane

3) and also that different batches of the purified antibody obtained after several uses of a single column maintain an invariable titer (see Electronic Supplementary Material, Fig. S1). Therefore, this method could be a useful and valuable tool to purify antibodies and also to detect protein–protein interactions between a cross-linked GST fusion protein and a mixture of proteins contained on a crude extract of any tissue.

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