

Immobilization of acidic polysaccharides on Sepharose for the isolation of specific antibodies

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Materials and Methods

DMSO (cat. No. 1.02931) was purchased from Merck KGaA (Darmstadt, Germany). *N,N'*-Disuccinimidyl carbonate, *N,N*-diisopropyl-*N*-ethylamine, and ethanolamine were from Sigma-Aldrich (Saint Louis, Missouri, USA). Amino Sepharose 6 FF was from GE Healthcare BioSciences AB (Uppsala, Sweden). Hyaluronic acid (15–30 kDa, Na salt) was from Contipro (Czech Republic). Polysaccharides PS15031 and PS15125 were isolated from the shoot cell wall of eelgrass *Zostera marina* L. and from the inflorescences of common tansy *Tanacetum vulgare* L., respectively, were isolated according to the described methods.^{S1,S2} Mannan from *Saccharomyces cerevisiae* M7504 was from Sigma-Aldrich (Saint Louis, Missouri, USA). BODIPY amine was from Lumiprobe (Russian Federation). Dowex 50WX2 (200–400 mesh) ion-exchange resin was from Acros Organics (Belgium). Thin-layer chromatography (TLC) was performed using TLC Silica gel 60 F254 aluminum sheets (No. 1.05554.0001, Merck KGaA, Darmstadt, Germany) with detection by charring after soaking in 7% H₃PO₄. Glycan-PAA conjugates, glycan-free PAA, and L-Rha α -PAA-Sepharose adsorbent were sourced from GlycoNZ (Auckland, New Zealand). The pH of aqueous solutions was measured using pH indicator strips Pehanon (Macherey-Nagel, Germany) and Panpeha (Sigma-Aldrich, Germany).

Preparation of PS-Sepharose adsorbents

PS Prⁱ₂EtNH⁺ salt

A solution of PS Na-salt (2 mg) in 0.6 mL of 20% ethanol was slowly passed through a Dowex 50W X2 (200–400 mesh) column (0.2 mL, Prⁱ₂EtNH⁺ form), then eluted with 20% ethanol (2

mL). After addition of Pr^i_2EtN (1 μL), the eluate was evaporated and the residue was freeze-dried from water. It should be noted that aqueous solutions of the $\text{Pr}^i_2\text{EtNH}^+$ salts of hyaluronic acid, galacturonans PS15031, and PS15125 have a slightly acidic pH. An aqueous solution of hyaluronic acid $\text{Pr}^i_2\text{EtNH}^+$ salt (25 mg/mL) has a pH of approximately 5.5.

Activation of hyaluronic acid with DSC

To a solution of hyaluronic acid $\text{Pr}^i_2\text{EtNH}^+$ salt (15–30 kDa, 12.7 mg, ~ 24.9 μmol by disaccharide units, prepared as described above from 10 mg of sodium hyaluronate 15–30 kDa) in dry DMSO (480 μL ; DMSO was dried with freshly activated 4 $\text{\AA}$ molecular sieves), a solution of DSC (0.912 mg, 3.56 μmol) in DMSO (22.8 μL , 40 mg/mL) was added, and the solution was kept for 18 h at 22.5 $^\circ\text{C}$. After addition of BODIPY amine (0.76 mg, 1.78 μmol) in DMSO (150 μL , 5 mg/mL), the reaction mixture was kept for 1 h at 22.5 $^\circ\text{C}$. After addition of Pr^i_2EtN (10.7 μmol , 18.6 μL of 10% v/v in DMF), the reaction mixture was kept for 4 h at 22.5 $^\circ\text{C}$. The main part of the BODIPY amine remained unchanged (apparently, most of the DSC was hydrolyzed by traces of water in DMSO and the polysaccharide): TLC data: silica gel R_f = 0.38 ($\text{CHCl}_3/\text{MeOH}/\text{water}$ 2:6:1), ninhydrin-positive. The solution was diluted with three volumes (2 mL) of ethanol, and after addition of 4M aqueous NaOAc (12.5 μL , 50 μmol), the thickened solution was thoroughly stirred, diluted with EtOAc to 10 mL, and thoroughly stirred again. After 20 min, the mixture was centrifuged ($5000 \times g$ / 15 min). The precipitate was re-suspended in 10 mL of ethanol, thoroughly stirred, and centrifuged again ($5000 \times g$ / 15 min). This procedure was repeated twice more. The precipitate was dried *in vacuo*, dissolved in water, and freeze-dried. The yield of BODIPY-labeled HA 15–30 kDa Na salt was 9.9 mg. The BODIPY content in the conjugate, estimated by spectrophotometry ($\lambda_{\text{abs}} = 503$ nm, $\epsilon = 92,000$), was 0.03 μmol per 1 mg, which corresponds to one dye molecule per ~ 30 kDa of sodium hyaluronate, or one dye molecule per 75 carboxy groups.

Reaction of mannan with DSC under slightly acidic conditions

Mannan does not contain carboxyl groups and can be used to evaluate the reactivity of hydroxy groups under the same conditions in which the activation of hyaluronic acid $\text{Pr}^i_2\text{EtNH}^+$ salt with DSC was carried out. An aqueous solution of mannan (25 mg/mL) had a pH > 8 (it was obtained by alkaline extraction and contained residual Na salts). To provide the slightly acidic conditions used for the activation of hyaluronic acid $\text{Pr}^i_2\text{EtNH}^+$ salt with DSC, it was necessary to acidify the mannan solution in DMSO. *N*-Hydroxysuccinimide was chosen as the acidifying agent. It was determined how much *N*-hydroxysuccinimide needed to be added to mannan so that its aqueous solution (25 mg/mL) would have the same pH of 5.5 as the solution of hyaluronic acid $\text{Pr}^i_2\text{EtNH}^+$ salt (25 mg/mL). The same amount of *N*-hydroxysuccinimide was added to the mannan solution in DMSO before reaction with DSC. To a solution of mannan (4 mg) in DMSO (192 μL), *N*-hydroxysuccinimide (1.6 mg) was added. After the addition of DSC (0.364 mg in DMSO, 9.1 μL , 40 mg/mL), all procedures were performed in the same way as described above for hyaluronic acid. The content of the label (BODIPY) in the isolated product was one label per ~ 180 kDa, that is, six times less than for hyaluronic acid, despite the fact that mannan contains almost twice as many hydroxyl groups per unit mass as hyaluronic acid. This result suggests that under slightly acidic conditions, DSC reacts predominantly with carboxylic groups of acidic polysaccharides.

Activation of galacturonans PS15125 and PS15031 with DSC

To a solution of PS $\text{Pr}^i_2\text{EtNH}^+$ salt (obtained from 2 mg of Na salt) in dry DMSO (100 μL for PS15125 or 200 μL for PS15031), DSC (0.2 mg, 0.78 μmol) in DMSO (5 μL) was added, and the solution was kept for 18 h at 22–23 $^{\circ}\text{C}$.

Conjugation of the activated PS with Amino Sepharose 6FF

The solution of the activated PS was added to a suspension of Amino Sepharose 6FF (1 mL) in dry DMSO (total volume 1.5–2 mL) and thoroughly stirred. After addition of Pr_2^iEtN (4 μL , 23 μmol), the suspension was gently stirred (orbital motion shaker) for 2 h at room temperature. More Pr_2^iEtN (4 μL , 23 μmol) was added, and the mixture was kept for an additional 2 h. After this time, no PS was observed in the supernatant. It should be noted that PS not activated with DSC migrates from the solution to Amino Sepharose due to ionic interactions (polyanion–polycation); however, after the addition of Pr_2^iEtN as described above, it returns to the supernatant and is readily detected by TLC (TLC data: silica gel, eluent $\text{CHCl}_3/\text{MeOH}/\text{water}$ 20:6:1, $R_f = 0$, visualization by charring after treatment with 7% H_3PO_4). In the case of DSC-activated PS, it is not observed in the supernatant even after the addition of a large excess of Pr_2^iEtN . This indicates that activated PS binds to Amino Sepharose through the formation of a covalent bond. After addition of 4 M aqueous NH_4OAc (8 μL , 32 μmol), the suspension was gently stirred for 0.5 h at room temperature. The adsorbent was thoroughly washed on a filter with 25% ethanol and water, and then suspended in 4 mL of 0.5 M NaHCO_3 . Acetic anhydride (120 μL) was added, and the suspension was gently stirred for 1 h. A sample of the adsorbent (3 μL) tested with ninhydrin showed the absence of free amino groups. The adsorbent was thoroughly washed on a filter with water and 25% ethanol, and stored in 25% ethanol at 4 °C. The adsorbent contains approximately 2 mg of PS (calculated as PS Na salt) per 1 mL.

References

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