Temperature Effects in Boronic Acid - Lectin Affinity Chromatography (BLAC)

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Introduction

Glycosylation is one of the most thoroughly studied co- and post-translational modifications of proteins. Investigation of the glycoproteome, especially focusing on glycosylation changes due to various diseases, is of great research interest, in particular with respect to biomarker discovery. Selective glycoprotein enrichment is a crucial issue in the analysis of such complex biological samples as human serum. Automated boronic acid - lectin affinity chromatography (BLAC) is an excellent tool to enrich and reveal various classes of human serum glycoproteins for downstream processing of profiling and identification. In this paper the temperature dependency of glycoaffinity enrichment is discussed in the wide temperature range of 7-64°C. Wheat germ agglutinin (WGA) and boronic acid (BA) functionalized agarose beads were used in the study with model proteins of trypsin inhibitor (WGA specificity: binding N-acetyl glucosamine and sialo glycoconjugates), ribonuclease B (boronic acid specificity, Man(1-4) and Man(1-6) units) and myoglobin and lysozyme (non-glycosylated negative controls). Glycoprotein enrichment level at the different temperatures was determined microLC analysis with UV detection.

Methods

Glycoaffinity micropartitioning:
- Sample mixture (100 μL each experiment):
  - 10 mg/mL ribonuclease B
  - 10 mg/mL myoglobin
  - 1 mg/mL trypsin inhibitor (containing 8.25 mg lysozyme, Sigma-Aldrich)
- Stationary phases: 200 μL pipette tip format
  - 5 μL agarose beads with m-aminophenyl boronic acid
  - 5 μL agarose beads with wheat germ agglutinin
  - 2.5 μL agarose beads with WGA and 2.5 μL agarose beads with boronic acid
- Conditions:
  - Conditioning: water and 20 mM phosphate buffer (pH 2.85)
  - Binding: washing with binding buffer (50 mM tartaric acid, 1 mM CaCl2, 1 mM MgCl2, 0.55% NaN₃, pH 8.5)
  - Elution with 0.1 M HCl
- RP-HPLC analysis of affinity partitioning eluent:
  - Column: ZORBAX Eclipse XB-D-C18
  - Gradient: 20-60% acetonitrile containing 0.05% trifluoro acetic acid, in 20 minutes.
- The peaks were collected as shown in Figure 1 followed by MALDI-TOF-MS analysis using sinapic acid matrix.

Results

The extent of affinity chromatography based glycoprotein enrichment at different temperatures was determined by RP-HPLC analysis. Figure 1 shows the RP-HPLC trace of trypsin inhibitor (ovomucoid, type III from chicken egg white). From the two peaks present in the chromatogram (tR: 11.87 and 15.33 min) one exhibited very well the glycoaffinity stationary phase as depicted in the insets of 1A and 1B at the lower panel of Figure 1, whereas analyses of the collected fractions are shown after BLAC partitioning (panels 1A, 1B and 2; corresponding fractions are indicated in the upper panel). Quantification and MALDI-TOF-MS analysis of the collected fractions revealed that the less retained component (tR: 11.87 min) in the RP-HPLC chromatogram was lysozyme (present in 82.5%) while trypsin inhibitor was only present in 17.5% (data not shown).

Figure 2 shows the RP-HPLC analysis after glycoaffinity partitioning of the model protein mixture at different temperatures. Based on the peak distribution delineated in Figure 2, Figure 3 depicts the partitioned amounts of ribonuclease B, trypsin inhibitor and myoglobin in concentrations (Figure 3A) and percentages (Figure 3B) in regard to the original model protein mixture, respectively. Enrichment of ribonuclease B and trypsin inhibitor (ovomucoid) showed optimum performance at ambient temperature (16-20°C) for both the boronic acid and WGA as well as with the selected column (BLAC/WGA). Although, the recovery rate for trypsin inhibitor seems remarkably higher that of the ribonuclease B, the temperature dependent glycoaffinity performance had apparently very similar profiles for both proteins with single and with mixed stationary phase.

The non-glycosylated protein, myoglobin was chosen as a negative control as it does not appear to interact with the affinity partitioning phases evaluated. Indeed, myoglobin was not detected in the eluates at any temperatures but at the highest of 64°C, where it was found in the fractions of all partitioning phases. This observation suggests that high temperatures might induce non-specific interaction between the carrier material and myoglobin. Table 1 shows the myoglobin yields at 64°C.

On the other hand, lysozyme (the high level impurity in the Sigma trypsin inhibitor) was present in the majority of trypsin inhibitor containing partitioning fractions (compare traces in Figure 2 results with the elution of lysozyme observed in Figure 3B and lysozyme separately with the traces obtained with the sample mixture using WGA and BLAC stationary phases in Figure 2). Please note that lysozyme is not glycosylated. Moreover the amount of eluted lysozyme – unlike ovomucoid and ribonuclease B – is rapidly decreasing with elevating temperature. Therefore, colution of ovomucoid with lysozyme is probably due to a thermodynamically less stable secondary interaction between these two proteins then to direct binding with the affinity phases.

Conclusion

Our results suggested ambient partitioning temperature of 16-20°C to provide the best yield for BLAC based enrichment of the glycoproteins tested. At higher temperatures, possible non-specific interactions in the presence of myoglobin in the eluate at 64°C. On the other hand, at lower temperatures, secondary protein-protein interactions might occur evidenced by the lysozyme being present along with the ovomucoid in several elution fractions of the WGA and BLAC/WGA partitioning material. Its amount was rapidly decreasing with elevating temperature, although the single protein itself, as shown in Figure 1, did not have any affinity to BLAC stationary phase at room temperature. As a first approximation, we consider this to be due to a complexation phenomenon between trypsin inhibitor and lysozyme, resulting in associated binding and co-elution. This complex is apparently losing stability above ambient temperature. Therefore, in case of such complex samples as human serum, the optimal temperature for BLAC glycoaffinity partitioning the recommended temperature range would be 30-40°C, where the affinity of the stationary phase to the target glycoproteins is still satisfactory, but neither of the non-specific binding mechanisms interplays significantly.

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