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Short communication

Optimization of enzymatic desialylation of human serum transferrin

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Introduction

Transferrin (Tf) is one of the major iron transport proteins in the human organism. It is a highly glycosylated bilobal protein that can bind up to two iron(III) ions thus forming a distorted octahedral ternary complex with both the amino acids of the protein and the synergistic carbonate anion. (MacGillivray et al., 1998) Proper glycosylation of transferrin is critical for its proper function in the body; various pathological conditions are closely associated with unusual sialylation patterns. The presence of different transferrin glycoforms is the result of variability in the glycosylation of N-linked oligosaccharide chains. These glycan chains are bi- or trinatenary and bind to residues Asn-413 and Asn-611. (Fu and van Halbeek, 1992) Sialic acid is usually located at the outmost ends of these oligosaccharide chains. Deviations from the usual sialylation patterns are associated with various genetic disorders and chronic diseases. Currently, sialic acid-deficient transferrin levels are commonly used as biomarkers of chronic alcohol abuse. (Del Castillo Busto et al., 2005) To better understand the properties of asialotransferrin, the production and subsequent purification of the protein is required because the percentage of asialotransferrin in native human apotransferrin is low. Asialotransferrin is produced by the action of commercially available sialidase enzymes, which can be quite expensive. Therefore, optimization of the processes to produce asialotransferrin at a low cost is required. After enzymatic desialylation, additional chromatographic purification of asialotransferrin can be performed using the pH gradient method. (Friganović et al., 2021)

Materials and methods

Chemicals and instrumentation

The GlycoCleave® Immobilized Enzymes (GALAB Technologies) and immobilized SialEXO® Microspin columns (Genovis) were used for enzymatic desialylation of human apotransferrin (Biorbyt Ltd, cat. no. orb80927 or Sigma, cat. no. T3309). Tris (Amresco), sodium acetate trihydrate (Kemika), calcium chloride dehydrated (Fluka) were used.

Optimisation of enzymatic desialylation

Desialylation procedures were performed using two different immobilized sialidase enzymes; Glycocleave® and SialEXO®. Both products contain enzymes immobilised on agarose beads. Glycocleave® is sold as a slurry containing the beads, while the immobilized SialEXO® beads are contained in a plastic column. For desialylation using the Glycocleave® kit, the manufacturer recommends using 400 µL of slurry (containing 200 µL beads). The beads must be washed thoroughly several times with the working buffer (0.05 M NaOAc, 0.001 M $CaCl_2$, pH = 5.5). The protein is dissolved in the working buffer (recommended up to 2.5 mg/mL, adjusting the pH to 5.5 if necessary) and added to the beads. Desialylation is performed in 2 mL microcentrifuge tubes containing the protein solution and enzyme beads, which were rotated using a tube revolver (10 rpm) at t = 37° C for at least 6 hours. In our optimization experiments, we varied the acetate buffer concentration, protein concentration, and incubation time. For desialylation using the SialEXO® columns, the protein should be dissolved in 0.02 M Tris working buffer (recommended range 0.1-5 mg/mL, pH = 6.8) and added

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to the column, which was prewashed three times with 300 µL working buffer (centrifugation for 1 min at 200 RCF each time). The column should be incubated at room temperature with end-over-end mixing for 30 min. The desialylated protein is recovered by centrifuging for 1 min at 1000 RCF. The manufacturer states that a single SialEXO® column can be used for desialylation of up to 0.5 mg of protein in a 30 min period, indicating that the SialEXO® column is intended for single use. In our optimization procedure, we reused the SialEXO® columns multiple times and varied both the protein concentration and the time of desialylation.

Validation of the desialylation procedure

To confirm the satisfactory extent of desialylation, the collected protein samples were analyzed by FPLC. Isoelectric focusing was performed using the pIsep buffers (CryoBioPhysica). A single-step linear pH gradient was used. Isoelectric focusing was performed mainly in the range of pH = 7-5.75. For the experiments on the ÄKTA Purifier 10 instrument, the SOURCE^{TM} 15Q 4.6/100 PE anion exchange column was used, while for the experiments on the ÄKTA Start instrument, two or four HiTrap Q HP anion exchange columns connected in series were used. Samples that gave satisfactory results were sent for glycan analysis. The N-glycans were first cleaved by adding 1.2 U PNGase F (Promega, USA) and incubated overnight at 37 °C. Subsequent purification was performed via HILIC-SPE and the fluorescently labeled N-glycans were analyzed by H-Class UPLC (Waters, USA) using a BEH glycan chromatography column (Waters, USA). For some samples, the above UPLC characterization was also coupled with MS/MS analysis using the Synapt G2-Si ESI-QTOF-MS system (Waters, USA).

Results and discussion

For the desialylation performed with the GlycoCleave® kit, we increased the protein concentration from the recommended 2.5 mg/mL to 6.25 mg/mL. With the 800 µL volume used, this corresponds to 5 mg of transferrin per single desialylation cycle. Due to the increased concentration, a greater pH change (acidification) was observed at the end of the incubation period. Therefore, the amount of acetate was increased to 0.2 M, compared with the 0.05 M indicated in the manufacturer's instructions. The desialylation time was either 24 or 48 h. This is a significantly longer time frame than the 6 h indicated by the manufacturer. Glycan analysis results showed that this procedure removed >90% of the sialic acid content from the native protein. In the case of SialEXO® columns, we significantly increased the desialylation time from the recommended 30 min to 24 h. The protein concentration was also increased from the recommended 0.625 mg/mL to 2.5 mg/mL. Further increase in protein concentration gave unsatisfactory results. Glycan analysis results showed that the desialylated protein contained only ≈1% of the original sialic acid content (≈99% removed sialic acid content). This result was obtained by pooling the desialylated samples for the 19 consecutive 24-hour desialylation procedures using 2 SialEXO® columns in parallel. This means that the theoretical mass of transferrin that can be desialylated with a single SialEXO® column is ≥47.5 mg.

Conclusions

Optimization of desialylation processes was performed for 2 different commercially available sialidase enzymes. Increasing the protein mass per single cycle together with a drastic increase in reaction time gave the best results. The fact that we were able to produce nearly 50 mg of desialylated transferrin using a single SialEXO® column designed to produce only 0.5 mg of said protein is particularly intriguing.

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