Bioengineering

Research article

A thermal responsive affinity ligand for precipitation of sialylated proteins

Lindsay Arnold, Tian-Bo Yang, and Rachel Chen *

School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA30332-0100, USA

* Correspondence: E-mail: rchen@chbe.gatech.edu.

Abstract: We report here the development of a thermal responsive affinity ligand specific to sialic acid, sialic acid containing oligosaccharides, glycoproteins, and other sialylated glycoconjugates. The ligand is a fusion protein of 40 repeats of pentapeptide of an elastin like polymer (ELP) and the 21 kD sialic acid binding domain of a *Vibrio cholera* neuraminidase (VCNA). For cost-effective synthesis, the fusion protein was targeted to the periplasmic space of an *E. coli lpp* deletion mutant, resulting in its secretion to the growth medium. A pre-induction heat-shock step at 42 °C for 20 minutes was necessary to achieve high level expression of the ligand. Under optimized induction condition (18 °C, 0.1 mM IPTG and 48 hours of post-induction cultivation), the ligand was produced to about 100 mg/L. The ligand exhibited a transition temperature of 52 °C, which could be depressed to 37 °C with the addition of 0.5 M NaCl. Using fetuin as a model sialylated protein, the ligand was applied in an affinity precipitation process to illustrate its potential application in glycoprotein isolation. The ligand captured 100% fetuin from an aqueous solution when the molar ratio of ligand to fetuin was 10 to 1, which was lower than the expected for full titration of sialic acid on the glycoprotein by the lectin. Elution of fetuin from ligand was achieved with PBS buffer containing 2 mM sialic acid. To evaluate how protein and other contaminants influence the recovery of sialylated proteins, CHO medium was spiked into the fetuin solution. The predominant protein species in CHO medium was found to be albumin. Although its removal of over 94% was evident, purified fetuin contained some albumin due to its over-abundance. Additional experiments with albumin contaminant of varying concentrations showed that below 1 mg/L, albumin had no impact on the affinity precipitation, whereas above 10 mg/L, some albumin was co-purified with fetuin. However, even at 50 mg/ml, fetuin recovery yield remained high (about 90%) and about 95% of albumin was depleted with only one cycle of precipitation and elution. These results suggest that the ligand could

be used in initial capture of sialylated proteins and to remove over-abundant albumin contaminant. To the best of our knowledge, this is the first thermal responsive sialic acid binding protein developed with gene fusion technology.

Keywords: Albumin depletion; Glycoprotein purification; affinity precipitation; sialylated proteins; thermal-responsive lectin

1. Introduction

The area of downstream pharmaceutical purification stands to receive the greatest financial benefit with the development of an affinity chromatography alternative. Over 80% of the cost associated with therapeutic protein production is found in the downstream processing. Despite a projected 48% general expansion of mammalian and microbial product production, the improvements in downstream processing are incremental [1]. Standard, popular methods of purification often include scaled up versions of successful bench-top methods, such as affinity chromatography. However, post scale up, these methods often succumb to certain drawbacks, such as flow rate limitation, diffusion constraints, and high maintenance costs related to column fouling or ligand leaching [2,3]. To combat these disadvantages, affinity precipitation techniques are being investigated as alternatives that do not require column operations [4–8].

Previously, thermo-responsive affinity ligands targeting fucosylated glycoproteins were developed and successfully used in affinity precipitation for fucosylated proteins [8,9]. Sialylated proteins are another important type of glycoprotein. One well-known example is Erythropoietin (EPO), the recombinant version of which is manufactured by Chinese hamster ovary (CHO) cell culture technology [10–12]. In an effort to expand the platform of using thermoresponsive lectins to sialylated glycoproteins, we designed a new sialic acid lectin and investigated its utility in affinity precipitation. We report here the cost-effective synthesis of the ligand, its characterization using fetuin as a model sialylated protein and additional experiments carried out to assess the influence of albumin contaminants on the precipitation process.

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise specified and were used without further purification. CHO cells and accompanying media was generously cultured and donated by Travis Meyer and Dr. Bao's lab from Georgia Institute of Technology. CHO-K1 (ATCC CCL-61) cells were cultured in 1x F-12 Media (with L-glutamine) prepared as 10% FBS CHO Growth Media. Cells were harvested and the remaining spent media was used for subsequent experiments.

2.2. Bacterial strains and plasmids

Escherichia coli strain K12 UT5600 (F- ara-14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1) was used as a host for all cloning steps. Plasmid pQTAT containing an N-terminal TAT signal sequence was used as the expression vector [13]. The artificial gene comprised of the lectin (*nanH* GenBank: M83562.1), a 10 amino acid glycine rich linker, and 20 VPGVG repeats was synthesized by GenScript (Piscataway Township, NJ, USA). The ELP segment with 20 repeats of pentapeptide was oligomerized to create a 40 pentamer ELP which was subsequently fused with the same linker and lectin. The synthetic genes and the expression vector pQTH were digested with BamHI and HindIII restriction enzymes (NEB), and ligated to form the plasmids for fusion expression, pQTVCNA40. The complete sequence of fusion protein is provided in Figure S1.

2.3. Fusion expression

This fusion protein (VCNA-ELP40) was expressed in an *E. coli* host. A single colony of *E. coli* 609Y/pQTVCNA40 transformant was inoculated into 3 mL LB broth and 100 µg/mL ampicillin and was cultivated overnight at 37 ˚C and 250 RPM. This seed culture was used to inoculate 100 mL LB broth culture containing 100 µg/mL ampicillin to an OD600 of 0.1. This larger culture was grown at 37 ˚C and 250 RPM to a cell density of OD600 of about 0.5–0.6, at which point the flasks were subjected to a heat shock of 42 ˚C for 20 minutes. The cultures were then allowed to cool to room temperature for 10 minutes before the addition isopropyl-β-D-thio-galactoside (IPTG) to a final concentration of 0.1 to 1 mM, to induce the production of the fusion protein. The cells were cultivated at 18 ˚C for an additional 48 hours .

The extracellular fraction was collected as the supernatant after pelleting the cells by centrifugation at 6,000 g at 4 ˚C for 25 min. This was repeated until supernatant was clear.

2.4. Glycoprotein isolation with inverse temperature cycling (ITC)

For solutions containing VCNA-ELP40 ligand bound to the target glycoprotein, the isolation via precipitation was performed using inverse temperature cycling. The solution was heated to the transition temperature of the ELP ligand in an Eppendorf Thermomixer for 5 minutes shaken at 300 RPM. The solution was then centrifuged at 16,000 x g for 1 minute. The supernatant was removed, and the pellet resuspended in a phosphate buffered saline (PBS) elution buffer containing 2 mM sialic acid. The resuspended solution was chilled at 4 ˚C for 30 minutes to maximize resolubilization of the ELP constructs. This solution was then subject to the same heating conditions to precipitate the fusion ligand, and centrifuged again for 5 minutes at 16,000 x g. The pellet contains isolated fusion ligand, and the supernatant was collected as the purified protein fraction.

2.5. Turbidity measurements

The transition temperature of the constructs was determined spectrophotometrically in a 96-well microplate reader (Spectramax M5, Molecular Devices, Sunnyvale, California). Each well contained

200 µL of PBS with 0 or 0.5 M NaCl with ligand fusion at a concentration of 5 mg/mL. Temperature was increased (in 2 ˚C increments) from 22 to 60 ˚C and the absorbance at 310 nm was measured.

2.6. SDS-PAGE

Each sample was combined in a 1:1 ratio with SDS sample buffer (10% SDS, 10% β-mercaptoethanol, 0.3 M Tris-HCl (pH 6.8), 0.05% bromophenol blue, 50% glycerol), boiled for 5 minutes, and resolved by 12.5% (w/v) SDS–PAGE. Each gel was detected by Coomassie blue staining (Bio-Rad).

2.7. Protein quantification

The soluble protein concentration was measured using the Bradford assay with Bio-Rad reagent (Bio-Rad, Hercules, USA) and bovine serum albumin as a standard.

3. Results

3.1. Construction of VCNA-ELP40 ligand

The *Vibrio cholera* neuraminidase (VCNA) is comprised of three distinct domains: a neuraminidase catalytic domain and two flanking lectin regions [14]. Of the two lectin domains, the N-terminal lectin has been found to bind with high affinity to sialic acid (Kd of 30 µM) and similar affinity to sialic acid containing substrates, such as α-2,3-sialyllactose and α-2,6-sialyllactose [15]. This 21 kD binding site is efficient in targeting terminal sialic acid moieties and does not require the addition of metal ions for binding. In the same manner as previous work with a fucose binding lectin, this lectin domain of the gene was C-terminally fused to a glycine rich linker connecting the gene encoding the $(VPGVG)_{40}$ sequence of the ELP domain [8]. In addition to these functional regions, an N-terminal TAT signal sequence was included so as to allow for secretion and easy isolation of the fusion construct after expression in *E. coli*. This signal sequence, when used in tandem with an *E. coli* mutant carrying a deletion of the *lpp* encoded lipoproteins, results in increased membrane permeability, allowing the translocated protein to leak into the growth medium [13]. The fusion ligand was termed VCNA-ELP40.

3.2. Production of ligand

Several parameters critical to the ligand expression, including temperature, inducer concentration and induction duration, were investigated to identify optimal conditions for ligand production. A cultivation temperature of 18 ˚C was found to be better than higher temperature used (30 ˚C or 37 ˚C). Two different IPTG concentrations (0.1 mM and 1 mM) were tested, and the total protein and recombinant protein levels secreted to the growth medium were monitored at 24 and 48 hours. The results are tabulated in Table 1.

It was concluded that a 48 hour, 18 ˚C cultivation after induction with 0.1 mM IPTG resulted in the best production levels of the VCNA-ELP40 fusion construct in E609Y. By utilizing this secretion

pathway, homogenization steps are avoided, and one-step isolation of the product is possible by simple temperature cycling. This precipitates the construct out of the culture media to be used for a purification scheme.

Table 1. Protein levels at 18 ˚C cultivation.

A. Total Protein (mg/L)		
	0.1 mM IPTG	1.0 mM IPTG
24 hours	370 ± 38	220 ± 19
48 hours	510 ± 52	330 ± 30

B. VCNA-ELP40 (mg/L)		
	0.1 mM IPTG	1.0 mM IPTG
24 hours	80 ± 8	40 ± 4
48 hours	100 ± 10	70 ± 7

3.3. Ligand characterization

The VCNA-ELP40 fusion construct was tested for its designed dual functionality. First, the ELP domain, which confers inverse temperature solubility to the ligand, was monitored by observing turbidity as a function of temperature, as illustrated in Figure 1. The transition temperature is calculated at 50% of the max turbidity, which in this fusion case is about 52 ˚C. However, addition of NaCl at a concentration of 0.5 M effectively lowers the transition temperature to about 37 ˚C, a temperature at which most proteins are stable.
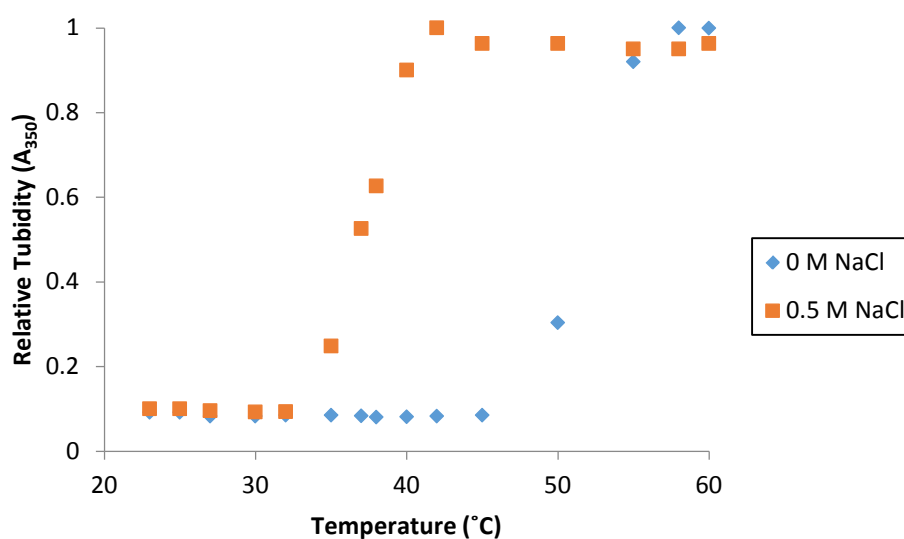

Figure 1. Turbidity profile to determine transition temperature of VCNA-ELP40.

Next, the lectin domain in the fusion protein was tested for recognition and binding of sialic acid on a glycoprotein. The model glycoprotein used here was fetuin, a sialylated protein. VCNA-ELP40 was added to a fetuin solution in a 2:1 molar ratio of lectin to glycoprotein and incubated at room temperature for 10 minutes. Thereafter, NaCl was added to the final concentration of 0.5 M and the temperature was raised to 40 ˚C. The precipitated protein complex was isolated from the supernatant, and the residual unbound fetuin in the supernatant is visualized in Figure 2 (Lane 2).

The precipitated protein complex was resuspended in an elution buffer containing 2 mM sialic acid to release the fetuin, and the precipitation process was repeated with salt and thermal stimulus. The precipitate, comprised of the VCNA-ELP40 ligand, was removed and is shown as Lane 3. The isolated fetuin in the resulting supernatant showed characteristic two close bands (Lane 5) similar to the pure form in the stock (Lane 1). This clearly shows that the lectin in the fusion was able to bind the sialic acid on fetuin and precipitated out during ITC, indicating the functionality of the lectin was retained in the ligand.
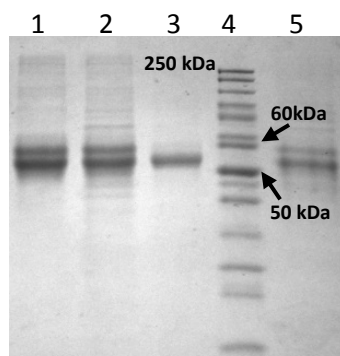

Figure 2. Lane 1: Fetuin stock solution Lane 2: Uncaptured fetuin after VCNA precipitation Lane 3: Isolated VCNA after purification Lane 4: MW ladder Lane 5: Isolated fetuin.

Not all fetuin was recovered in this preliminary test. In fact, a significant portion of fetuin was found in the unbound fraction (Lane 2, Figure 2). This was due to suboptimal molar ratio of ligand to target protein. Therefore, additional experiments were conducted to investigate a condition for complete recovery of fetuin. To this end, molar ratios of ligand to fetuin from 1 to 13 were tested to observe the change in recovery yield of fetuin. As seen in Figure 3, a complete recovery occurred around 10:1 VCNA-ELP to fetuin. Standard error for the recovery experiments performed in triplicate is plotted. Thus, by using sufficiently high ligands, complete recovery of a sialylated protein is possible.
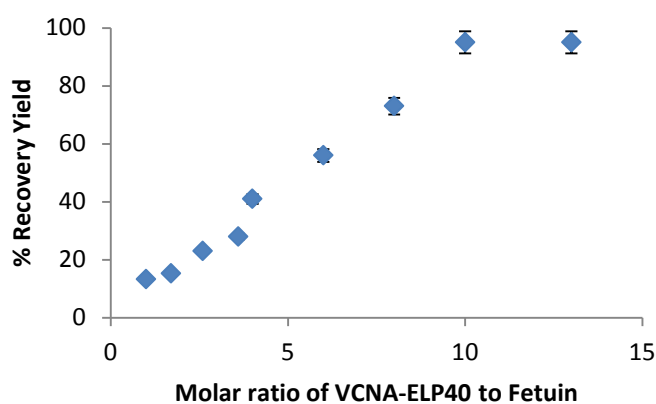

Figure 3. Recovery of fetuin as a function of molar ratio of ligand to target protein.

3.4. Effect of CHO growth medium on fetuin isolation yield

As sialylated proteins are produced predominately from CHO cell culture, it is of interest to evaluate the potential impact of medium components on purification process using the designed ligand. To this end, 18 µg of the model sialylated protein, fetuin, was spiked into 100 µL of CHO growth medium, to simulate an environment from which the model protein is to be isolated using affinity precipitation. VCNA-ELP40 was added in a ratio of 10:1 to the fetuin, and performed ITC using the optimal conditions (37 °C + 0.5 M NaCl) as described above. The elution of the fetuin from the ligand was done with an elution buffer containing 2 mM sialic acid in PBS, and the ligand was isolated with another round of ITC. The SDS-PAGE of the fractions is shown below in Figure 4.

As depicted in the SDS-PAGE in Figure 4, the VCNA-ELP40 was successfully isolated using the ITC and elution methods. No remaining fetuin is visible in the ELP ligand lane (lane 3). The CHO medium used was dominated by albumin. The unbound fraction is still predominantly albumin, with some variation of other contaminants seen at other molecular weights as well, suggesting that a majority of contaminant albumin could be removed by the simple precipitation process. The purified fraction, which ideally would contain only fetuin, still shows the presence of some albumin. Based off protein quantification, about $60 \pm 3\%$ of the fetuin was isolated. The estimated purity in the SDS-PAGE is about 70%. The amount of remaining albumin in the product stream was measured as well. The reduction of this abundant albumin ranges from 94–97% reduction from the original media. This is a promising way to reduce highly abundant proteins. Apparently, CHO medium does depress isolation yield quite significantly.
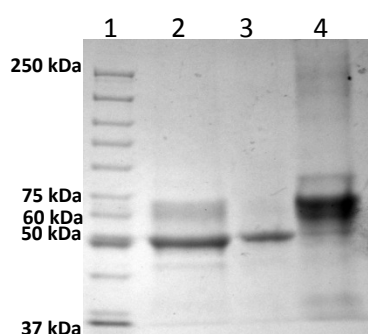

Figure 4. Fetuin precipitation from CHO cell media. Lane 1: MW marker Lane 2: Purified fetuin from spent CHO cell media Lane 3: Isolated VCNA affinity ligand after fetuin purification Lane 4: Remaining unbound fraction of CHO media after purification.

3.5. Effect of albumin on fetuin isolation yield and albumin depletion

The impact of albumin, the predominant protein components in CHO growth medium, on the affinity precipitation process, was further studied. Using bovine albumin as model contaminant, the above fetuin isolation experiments were repeated with albumin concentrations (0, 0.1, 1, 10, and 50 mg/mL). The final purified forms are depicted via SDS-PAGE analysis below in Figure 5.
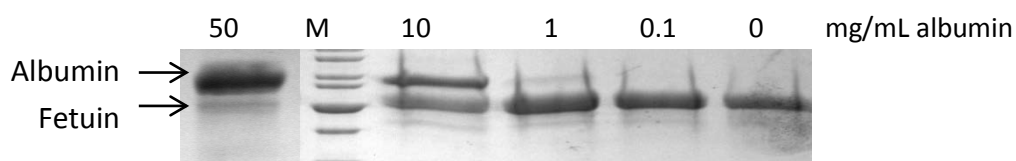
Figure 5. Purified fetuin from high albumin solutions. Reference band at 50 kDa.

From Figure 5, we can conclude that albumin at a low concentration (~ 1 mg/ml) would not impact the purity of the isolated glycoprotein. However, at a concentration of 10 mg/ml or above, significant amount of albumin was co-precipitated and co-purified with fetuin. The impact of albumin on isolation yield was minimal, as illustrated in Figure 6, as at its highest concentration tests, the isolation yield of fetuin remained high, 95%. This is much higher than the 60% yield from CHO medium described in the last section, suggesting that other components in CHO medium, possibly other sialylated proteins, had an impact on the isolation process.
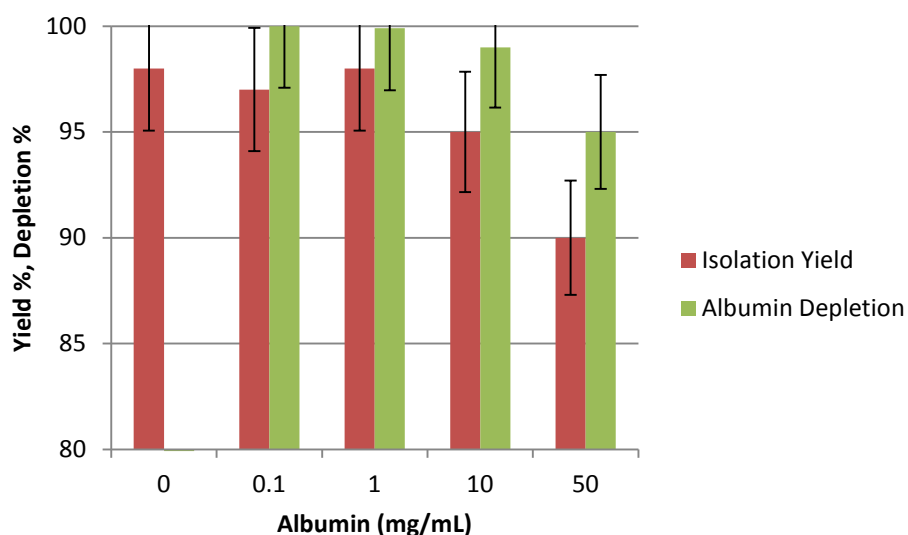

Figure 6. Isolation yield of fetuin and % albumin depleted as a function of albumin concentration. One standard deviation shown.

Also shown in Figure 6 are the depletion percentages of albumin, which is important for industrial purification of CHO derived glycoprotein as well as for processing of blood samples as they both contain large amounts of albumin. Albumin in blood samples range from 34–54 mg/mL, whereas the glycoprotein of interest often presents as a low abundant protein. Figure 6 shows that even at highest albumin tested concentration of 50 mg/ml, the removal of albumin was 95%, indicating that the affinity precipitation using the ligand developed could be potentially used as a method of albumin depletion in sample preparation.

4. Discussion

In this work, we developed the first sialic acid binding protein (VCNA-ELP40) that is capable of reversible phase transition in response to temperature changes. The dual functional protein is a fusion of the sialic acid lectin domain and the ELP, with the latter size chosen so that the transition temperature is within the range that most proteins are stable. The ligand is useful in isolation of sialylated proteins by a simple precipitation process above the transition temperature. As shown in this work, the recovery yield is dependent on the molar ratio of the ligand and the target sialylated protein, fetuin. Unlike other lectins, the sialic acid lectin domain of VCNA does not form multimers. Therefore, there is only one binding site per lectin. Fetuin has, on average, about 13.6 sialic acid residues per molecule [16]. Our previous studies showed that a complete capture of target fucosylated proteins require the fucose ligand be provided in quantities sufficient for the full titration of fucose on the target glycoprotein [8,9]. A complete titration of sialic acid binding sites by the ligand, VCNA-ELP40, is theoretically reached with a molar ratio of approximately 13:1 VCNA to fetuin. Our experiment value is 10, lower than the predicated value. The discrepancy could be simply due to the lower than expected sialylation level in our samples. Regardless, it is clear that a significant molar excess of the ligand is needed to achieve high or complete capture of target protein using the affinity precipitation process. Thus cost-effective production of the ligand is important. Interestingly, we found that high level expression can only be reached when a pre-induction heat shock is included. The exact reason is not known. Presumably, lectin domain expression requires certain host factors that are induced by heat-shock. Efforts toward optimization of induction conditions led to an expression level of 100 mg/L. The fusion protein was produced as an extracellular protein, thanks to the outer membrane mutation of the host strain, E609Y [13]. This greatly facilitates the isolation of the ligand as no cell breakage is needed. Additionally, the temperature responsive precipitation significantly simplifies the purification of the ligand. Together, the high expression and ease of isolation of purification ensure the cost-effectiveness of the ligand preparation.

As an alternative, the lectin portion could be immobilized on a matrix without the fusion to ELP. The resulting ligand can then be used in batch mode, similar to the affinity precipitation process as described here, but without the need of temperature shift. However, immobilization often leads to low yield and heterogeneity, and loss of ligand over repeated uses. The fusion production of lectin ligand is designed to overcome these disadvantages.

Glycoproteins are present in a complex mix. CHO medium is one example. The impact of isolation of fetuin from CHO medium was attempted and isolation yield of fetuin was clearly impacted by the numerous components in the complex medium. While pinpointing a specific component that caused the reduction in isolation yield is outside our research scope, we investigated how albumin, the most abundant protein in the medium, on the performance of the ligand. When albumin is the only contaminant, even at a large excess (relative to fetuin), we found the ligand performed well and the specific interaction between fetuin and ligand was not significantly impacted. As a result, the yield of sialylated protein recovery was above 90% while the removal of albumin was 95–99%. The overabundance of albumin impacted the quality of the isolated proteins. This is presumably due to the entrainment of albumin during precipitation. The trapped albumin could be easily removed if a wash procedure is included or another cycle of precipitation/elution is used.

Albumin is also present is large quantities in blood samples. Together with IgG, the two proteins represent 65% of the total protein in blood samples, overwhelming the low abundant proteins of significant diagnostic values. Thus, depletion of albumin is an important step in sample preparation. Of all the methods being utilized, the affinity methods were considered most effective [17,18]. These affinity methods rely on the binding to albumin, the high abundant protein. Thus they require significant amount of ligands, which are antibodies or other expensive reagents [17,18]. In comparison, depletion of albumin using the ligand we developed has two distinct advantages. First, since we target the minor component in the mixture, significantly less ligand is required. Second, the depletion and capture of glycoprotein is simultaneous, requiring no additional step or chemicals. Therefore, the ligand should be used for this purpose as well.

In summary, a novel sialic acid ligand was developed, along with a cost-effective method of its biosynthesis. The ligand can be used to isolate sialylated proteins in an affinity precipitation process. Additionally, the ligand could also be used in blood sample preparation to simultaneously capture sialylated proteins and deplete overabundant albumin.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

References

1. Lightfoot EN, Cockrem MCM (2013) Complex Fitness Diagrams: Downstream Processing of Biologicals. *Sep Sci Technol* 48(12): 1753–7.
2. Jungbauer A (1993) Review: Preparative chromatography of biomolecules. *J Chromatogr* 639: 3–16.
3. Patchornik G, Albeck A (2005) Free nonimmobilized ligands as a tool for purification of proteins. *Bioconjug Chem* 16(5): 1310–5.
4. Meyer DE, Chilkoti A (1999) Purification of recombinant proteins by fusion with thermally-responsive polypeptides. *Nat Biotechnol* 17: 1112–5.
5. Linné-Larsson E, Galaev I, Lindahl L, et al. (1996) Affinity precipitation of Concanavalin A with p-aminophenyl- α-D-glucopyranoside modified Eudragit S-100. *Bioseparation* 6: 273–282.
6. Liu F, Tsai SL, Madan B, et al. (2012) Engineering a high-affinity scaffold for non-chromatographic protein purification via intein-mediated cleavage. *Biotechnol Bioeng* 109: 2829–35.
7. Fong B, Wu W, Wood DW (2009) Optimization of ELP-intein mediated protein purification by salt substitution. *Protein Expr Purif* 66: 198–202.
8. Arnold L, Chen R (2014) Novel thermo-responsive fucose binding ligands for glycoprotein purification by affinity precipitation. *Biotechnol Bioeng* 111(2): 413–7.
9. Arnold L, Chen R (2015) One-step non-chromatography purification of a low abundant fucosylated protein from complex plant crude extract. *AIMS Bioeng* 2: 249–263.

10. Sethuraman N, Stadheim T (2006) Challenges in therapeutic glycoprotein production. *Curr Opin Biotechnol* 17(4): 341–6.
11. Misaizu T, Matsuki S, Strickland TW, et al. (1995) Role of antennary structure of N-linked sugar chains in renal handling of recombinant human erythropoietin. *Blood* 86(11): 4097–104.
12. Skibeli V (2001) Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin. *Blood* 98(13): 3626–34.
13. Shin H-D, Chen RR (2008) Extracellular recombinant protein production from an Escherichia coli lpp deletion mutant. *Biotechnol Bioeng* 101(6): 1288–96.
14. Crennell S, Garman E, Laver G, et al. (1994) Crystal structure of Vibrio cholerae neuraminidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure* 2(6): 535–44.
15. Moustafa I, Connaris H, Taylor M, et al. (2004) Sialic acid recognition by Vibrio cholerae neuraminidase. *J Biol Chem* 279(39): 40819–26.
16. Spiro RG (1960) Studies on fetuin, a glycoprotein of fetal serum. *J Biol Chem* 235: 2860–9.
17. Eriksson C, Schwenk JM, Sjoberg A, et al. (2011) Affibody molecule-mediated depletion of HAS and IgG using different buffer compositions: a 15 min protocol for parallel processing of 1-48 samples. *Biotechnol Appl Biochem* 56: 49–57.
18. Haudenschild DR, Eldridge A, Lein PJ, et al. (2014) High abundant protein removal from rodent blood for biomarker discovery. *Biochem Biophys Res Commun* 455: 84–9.

Supplementary

VCNA-ELP40 amino acid sequence

G S A L F D Y N A T G D T E F D S P A K Q G W Met Q D N T N N G S G V L T N A D G Met
P A W L V Q G I G G R A Q W T Y S L S T N Q H A Q A S S F G W R Met T T E Met K V L S G
G Met I T N Y Y A N G T Q R V L P I I S L D S S G N L V V E F E G Q T G R T V L A T G T A
A T E Y H K F E L V F L P G S N P S A S F Y F D G K L I R D N I Q P T A S K Q N Met I V W
G N G S S N T D G V A A Y R D I K F E I Q G D E L G G G G S G G G G S G G G G S G G G G
S V D V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G
V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G
V G V P G V G V P G V G V P G V G L D V P G V G V P G V G V P G V G V P G V G V P G V
G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P
G V G V P G V G V P G V G V P G V G V P G V G V P G V G V P G V G