Glycoconjugate Journal 16, 719–723, 1999

Triazine dyes as inhibitors and affinity ligands of glycosyltransferases

Joanna Kamińska, Jacek Dzięcioł and Jerzy Kościelak*

Department of Biochemistry, Institute of Hematology and Blood Transfusion, Chocimska 5, 00-957 Warsaw, Poland

The triazine dyes: Cibacron Blue 3GA, Reactive Red 120, Reactive Yellow 86, Reactive Green 19, Reactive Blue 4, Reactive Brown 10 inhibited the activity of a purified preparation of α1,6fucosyltransferase (GDP-L-fucose : N-acetyl β-glucosaminide 6-α-L-fucosyltransferase, EC 2.4.1.68) from human blood platelets. Cibacron Blue 3GA and Reactive Red 120 were examined for the nature of the inhibition and both were found to be competitive inhibitors of the enzyme, with $K_i = 11$ μM and 2 μM, respectively. The two dyes inhibited also serum glycosyltransferases: α1,2fucosyltransferase (GDP-L-fucose: β-D-galactosyl-R2-α-L-fucosyltransferase, EC 2.4.1.69), β1,4galactosyltransferase (UDP-galactose: N-acetyl-D-glucosamine 4-β-D-galactosyltransferase, EC 2.4.1.90) and β1,3N-acetylglucosaminyltransferase (UDP-GlcNAc: 4-β-D-galactosyl-D-glucose). Cibacron Blue 3GA was a more effective inhibitor of the glycosyltransferases that use UDP-linked sugar donors than Reactive Red 120 while the latter was a stronger inhibitor of the fucosyltransferases that use GDP-linked donor. All four glycosyltransferases could be affinity purified on Cibacron Blue 3GA-Agarose columns. The order of elution of glycosyltransferases from the columns with solutions of 0.25–1.0 M potassium iodide also depended upon the structure of nucleotide sugar donor, i.e. whether it contained UDP or GDP. Thus, triazine dyes should interact with the sugar donor binding sites of glycosyltransferases. The main advantages of the use of triazine dyes as affinity ligands for isolation of glycosyltransferases are their universal applicability regardless of enzyme specificity, low cost, and insensitivity to high concentration of other proteins present in the solution.

Keywords: **fucosyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, inhibitor, triazine dye, purification**

Most glycosyltransferases that carry out biosynthesis of glycosidic linkages use nucleotide sugar phosphates as donor substrates [1]. Isolation of these enzymes is difficult because they are present in cells and body fluids only at low concentrations. Glycosyltransferases are usually purified by affinity chromatography employing immobilized analogues of either donor or acceptor substrates. Triazine dyes, polysulphonated aromatic chromophores, mimic the naturally occurring biological heterocycles such as nucleotide mono-, di- and triphosphates, NAD+, NADH+, flavins, acetyl-CoA and folic acid [2,3] and inactivate typical nucleotide-dependent enzymes with different efficiency [2,4]. Thus, they can be used as affinity ligands in procedures aimed at purification of glycosyltransferases. They are being used, however, for this purpose infrequently [5,6,7] and the kinetics of glycosyltransferases have never been studied in the presence of triazine dyes. Herein we report a quantitative study on inhibition and affinity chromatography of four glycosyltransferases employing triazine dyes, with a special emphasis on α1,6fucosylsyltransferase (α1,6FucT) of human platelets. The latter enzyme may perform in platelets some special function since it is released from these cells during blood coagulation [8,9] and its activity within platelets is probably a measure of the ploidy level of megakaryocytes [10].

*To whom correspondence should be addressed: Prof. Dr Jerzy Kościelak, Institute of Hematology and Blood Transfusion, Department of Biochemistry, ul. Chocimska 5, 00-957 Warsaw, Poland; Tel./Fax: (48-22) 848-95-15; Fax: (48-22) 848-89-70;
E-mail: kosci@atos.warman.com.pl

Materials and methods

Materials

GDP-L-[^{14}C]fucose, UDP-D-[^{14}C]galactose and UDP-D-[^{14}C]N-acetylglucosamine were obtained from Amersham (Buckinghamshire, UK). PBE 94, polybuffer 96, CH-activated Sepharose 4B and Sephadex G-200 were purchased from Pharmacia (Uppsala, Sweden). Ethanolamine, Triton CF 54, human apo-transferrin, glycerol, EDTA, phenylmethylsulfonyl fluoride (PMSF), Cibacron Blue 3GA (CB 3GA), Reactive Red 120 (RR 120), Reactive Yellow 86, Reactive Green 19,

Reactive Blue 4, Reactive Brown 10, Cibacron Blue 3G-A-Agarose (CB 3GA-A) as well as reagents and protein standards for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Sigma (Deisenhofen, Germany). GDP-hexanolamine-Sepharose was a kind gift from Dr M. Błaszczyk. Partially purified α1,6FucT (up to the Sephadex G-200 step) was obtained by the method of Kamińska et al. [11].

Glycosyltransferase Assays

Activity of α1,6FucT (GDP-L-fucose : N-acetyl β-glucosaminide 6-α-L-fucosyltransferase, EC 2.4.1.68) from human platelets and blood serum was determined with asialo-agalacto-glycopeptide from human transferrin [11] that should be a specific substrate for the enzyme [12]. Activity of α1,2FucT (GDP-L-fucose: β-D-galactosyl-R 2-α-L-fucosyltransferase, EC 2.4.1.69) from human serum was determined with β-phenyl-galactoside as substrate [13]. The latter was not an acceptor when sera from "Bombay" or para "Bombay" donors (that did not contain α1,2-fucosyltransferase activity) were used as enzyme source. Activity of serum β1,4GalT (UDP-galactose: N-acetyl-D-glucosamine 4-β-D-galactosyltransferase, EC 2.4.1.90) was determined employing free GlcNAc as substrate [14]. Activity of serum β1,3GlcNAcT (UDP-GlcNAc: 4-β-D-galactosyl-D-glucose) was assayed under condition when 78% and 22% of the transferred [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc was conveyed to carbon atom 3 and 6 respectively of galactose residue of lactose [15]. Thus, the activity determined by the method is preferentially that of β1,3GlcNAcT.

Preparation of Asialo-agalactotransferrin-glycopeptide as Substrate, and Affinity Column Chromatography

Asialo-agalactotransferrin-glycopeptide (GnGn-Gp) was prepared from apo-transferrin as described previously [16] and used either as a substrate for determination of α1,6FucT activity or (after coupling to CH-Sepharose 4B) for purification of the enzyme by affinity chromatography [17].

Cibacron Blue 3GA-Agarose Chromatography

Chromatography of glycosyltransferases on CB 3GA-A was performed as follows: human blood serum (1 ml) was applied to CB 3GA-A column (1.2 cm × 1.8 cm) previously equilibrated with 50 mM cacodylate buffer, pH 7.0, containing 40 mM $MgCl_2$, and 10% glycerol (Buffer A). The unbound material was washed with Buffer A. The column was subsequently eluted with Buffer A without $MgCl_2$ but containing increasing concentrations of potassium iodide: 0.25, 0.5, 1.0 M KI, Enzyme-containing fractions were collected and dialyzed for 20 h against Buffer A without $MgCl_2$.

SDS-PAGE

This was performed according to Laemmli [18], with the use of 10% acrylamide. Staining of the gels with silver nitrate followed the standard protocol of Bio-Rad (Hercules, CA, USA).

Results and Discussion

Inhibition of glycosyltransferases by triazine dyes. Figure 1 shows the results of inhibition of a partially purified α1,6FucT by each of the six triazine dyes examined. The strongest inhibitors were RR 120, Reactive Brown 10, and CB 3GA. RR 120 and CB 3GA were selected for further studies and tested for the nature of inhibition employing Dixon plot [19]. Both were found to be competitive inhibitors of α1,6FucT with K_i for CB 3GA = 11 μM and that for RR 120 = 2 μM. In addition, CB 3GA and RR 120 were examined as inhibitors of three glycosyltransferases from human blood serum namely α1,2fucosyltransferase (α1,2FucT), β1,4galactosyltransferase (β1,4GalT), and β1,3N-acetylglucosaminyltransferase (β1, 3GlcNAcT) (Fig. 2B, C, D respectively). When concentrations of the dyes were plotted against activities of these glycosyltransferases including α1,6FucT (Fig. 2A) we found that the plots differed in shape attesting to the complexity of the dye-enzyme binding. This presumably resulted from a unique combination of ionic and hydrophobic sites in each partner of the reaction. Nevertheless, some regularity was observed in that RR 120 inhibited fucosyltransferases that use GDP-linked donor more strongly than did CB 3GA whereas the reverse was true for glycosyltransferases that use UDP-linked donors. It is to be noted that the triazine dyes inhibited the activity of α1,6FucT at 100 times lower concentration than the activity of the other glycosyltransferases. The difference is likely due to the fact that in the first instance, we used a partially purified

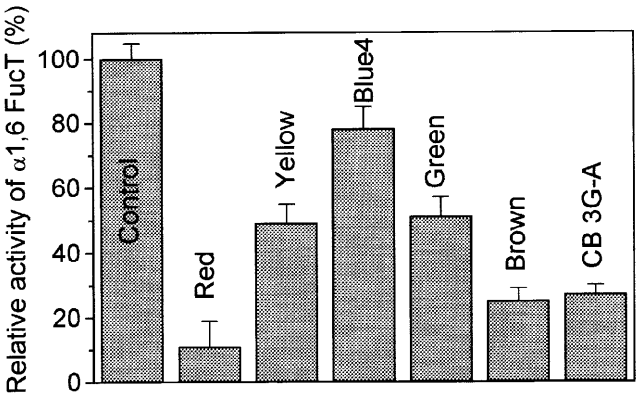

Figure 1. Inhibition of a partially purified α1,6fucosyltransferase from human platelets by different triazine dyes (Reactive Red 120, Reactive Yellow 86, Reactive Blue 4, Reactive Green 19, Reactive Brown 10, Cibacron Blue 3GA). Triazine dyes were used at 50 μg/ml concentration. Data are the means ± SD of three independent experiments.

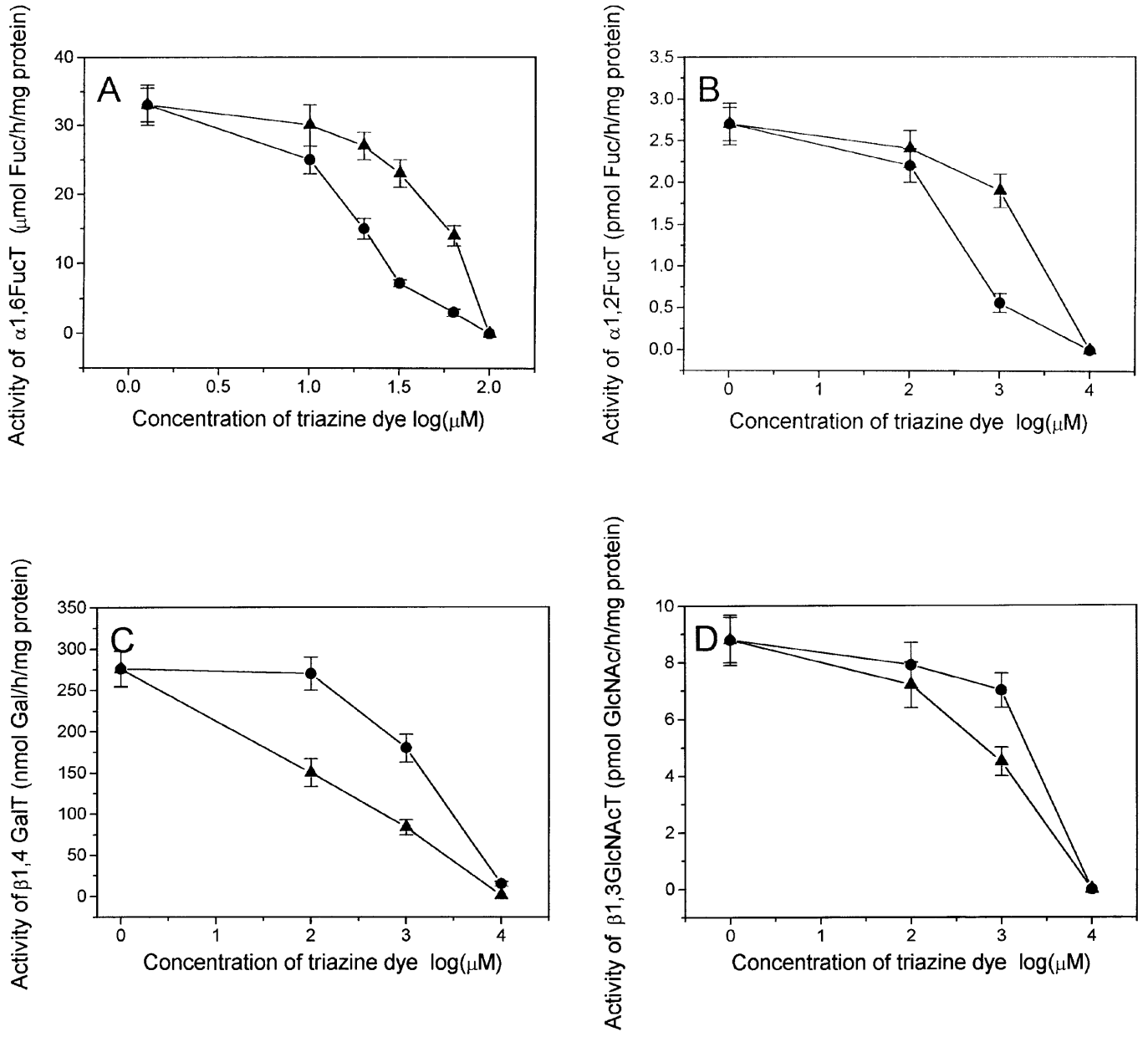

Figure 2. Inhibition of glycosyltransferases by Cibacron Blue 3GA (▲) and Reactive Red 120 (●). (A), α1,6fucosyltransferase partially purified from human platelets, (B), α1,2fucosyltransferase from human serum, (C), β1,4galactosyltransferase from human serum; (D), β1,3N-acetylglucosaminyltransferase from human serum. Data are the means ± SD of three independent experiments.

enzyme whereas in the other three—whole human serum was employed as enzyme source.

The high inhibitory activities of CB 3GA and RR 120 towards glycosyltransferases strongly suggested that both dyes might be used as effective ligands for affinity chromatography of the enzymes. Therefore we examined in this role CB 3GA linked to agarose, which is commercially available and widely used for protein separation. Presence in the buffer of 0.1% Triton CF-54 weakened the binding and precluded satisfactory separation. For elution of the columns we initially employed 0.5–2.0 M NaCl or 0.5–2.0 M $(NH_4)_2SO_4$ but obtained only a very small recovery of protein and enzyme activity in the eluates. A much more effective elution could be achieved with a chaotropic agent KI in the 0.25–1.0 M concentration range. Similar observations were made during chromatography of mitochondrial anion carriers on CB F3GA-Sepharose [20]. Interestingly, we found that the activities of both α1,2FucT and α1,6FucT were eluted from CB 3GA-A columns with 0.5 M KI (Fig. 3A and B) whereas those of β1,4GalT and β1,3GlcNacT appeared mainly in 0.25 M KI eluates (Fig. 3C and D). Thus,

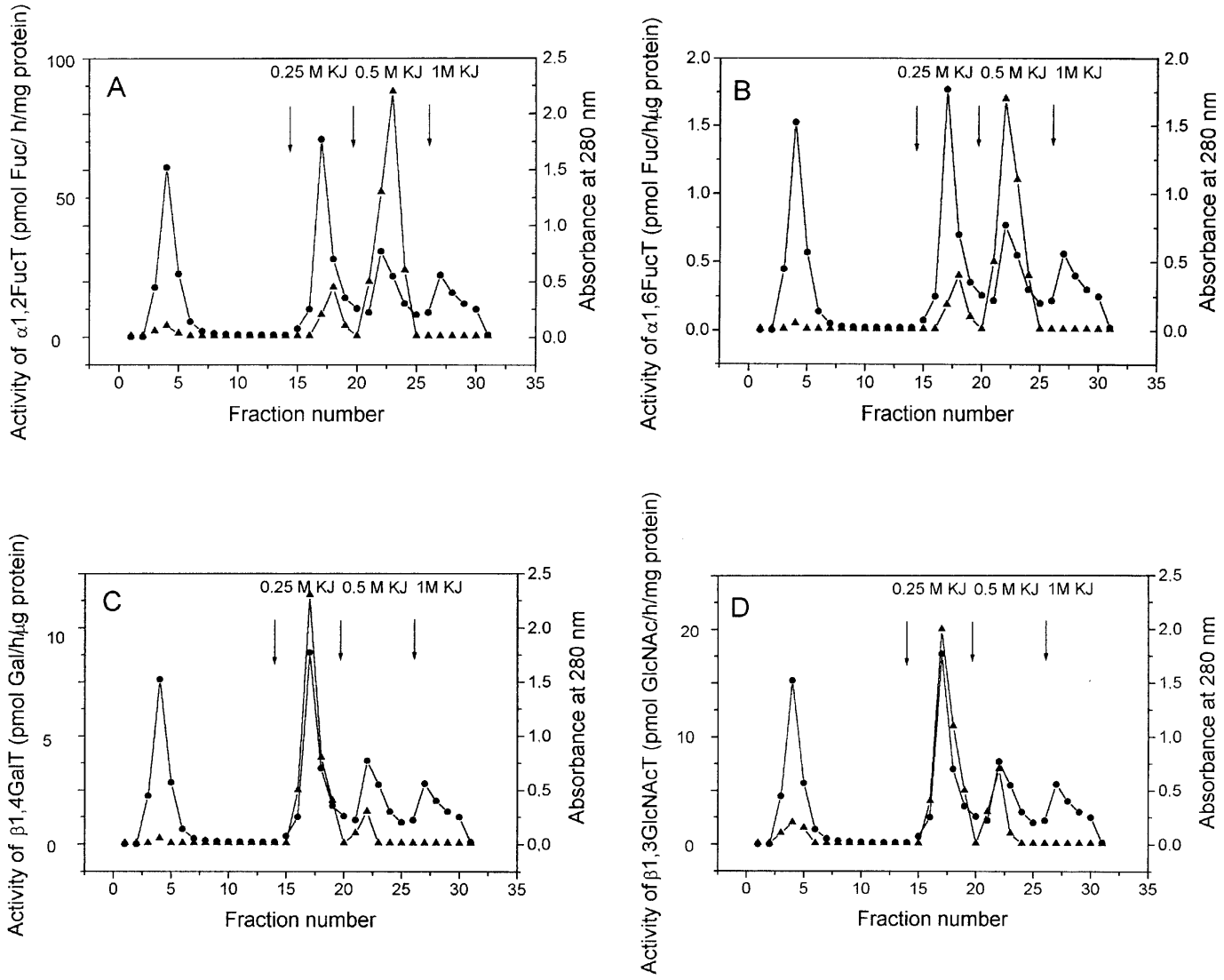

Figure 3. Gradient elution with aqueous solution of KJ of serum glycosyltransferases from Cibacron Blue 3GA-Agarose columns. (A), α1,2fucosyltransferase; (B), α1,6fucosyltransferase; (C), β1,4galactosyltransferase; (D) β1,3N-acetylglucosaminyltransferase. The serum was applied as described in "Materials and Methods". The KJ pulse is indicated by the arrow. Fractions (volume = 2 ml) were assayed for the enzyme activity (▲) and protein concentration (●).

the elution of glycosyltransferases from CB 3GA-A columns, similarly as the inhibition potency, depended largely on the structure of the nucleotide, i.e. whether it was GDP or UDP. Recovery of enzyme activities and purification were in all instances similar amounting to 80% and 8-fold, respectively. These results also show that separation of glycosyltransferases can be obtained even when whole serum is applied to CB 3GA-A which means that the dye is insensitive to high concentration of other proteins present in the solution.

Finally, we compared the efficacy of chromatography of a partially purified α1,6FucT on CB 3GA-A with that on GnGn-Gp-CH-Sepharose, after the chromatofocusing step [11]. Recoveries of enzyme activities were 80% and 56% with a 10-fold and 9-fold purification for the former and the latter method, respectively. Protein composition of enzyme preparations obtained by the two methods is shown in Figure 4. Except for the presence of α1,6FucT, they are quite different with respect to contaminating proteins and, thus, the two methods can be used in succession.

Sticher et al. [5] used CB F3GA-Sepharose for purification of sialyltransferase from human liver. The adsorbed transferase was subsequently eluted with a linear NaCl gradient (0–2 M NaCl). The yield was 82% with an 8.3-fold purification. Cibacron Blue was also used for purification of dolichol phosphoryl mannose synthase from Thermoplasma acidophilum [6], and of UDP-N-acetylgalactosamine : polypeptide N-acetylgalactosaminyltransferase from human placenta [7]. In the first instance the enzyme was eluted with a 0–4 M NaCl

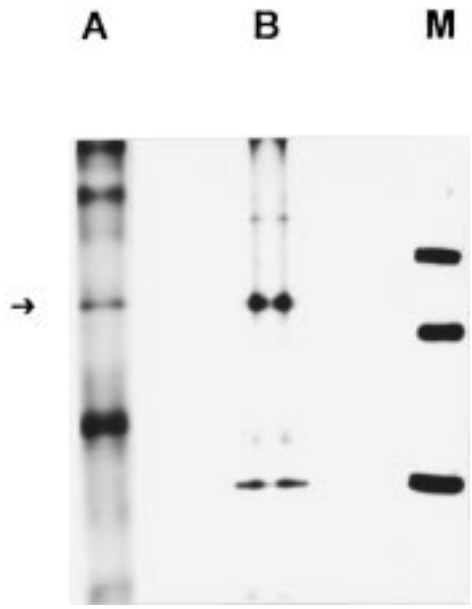

Figure 4. SDS-PAGE profile of partially purified α1,6fucosyltransferase from human platelets. Electrophoresis was performed in 10% polyacrylamide gel under non-reducing conditions. Fractions obtained at two different steps of the purification procedure were stained with silver nitrate. (A), Fraction eluted from GnGn-Gp-Sepharose 4B column; (B), fraction eluted from Cibacron Blue 3GA-Agarose column; (M), marker proteins from top to bottom: bovine serum albumin (66,000), fumarase (48,500) and carbonic anhydrase (29,000). α1,6Fucosyltransferase is indicated by the arrow.

gradient and in the second with 1.5 M KCl. The recovery and purification were lower than in case of sialyltransferase. Thus, the chromatography on Cibacron Blue appears to be a universal procedure applicable for purification of glycosyltransferases regardless of their specificity. Additional advantages of the procedure are low cost and commercial availability of the ligand as well as its insensitivity to high concentration of other proteins present in the solution.

Acknowledgments

This paper was in part supported by a grant no. 4P05A02016 from the Committee for Scientific Studies in Poland.

References

1 Schachter H (1995) In *Glycoproteins* (Montreuil J, Schachter H, Viegenthart JFG, eds.) pp 123–124. Elsevier Science B.V.
2 Clonis D, Lowe R (1980) *Biochem. J.* **191:** 247–51.
3 Hanggi D, Carr P (1985) *Anal. Biochem.* **149:** 91–104.
4 Dean PDG, Watson DH (1979) *J. Chromatogr.* **165:** 301–19.
5 Sticher U, Gross HJ, Brosmer R (1991) *Glycoconj. J.* **8:** 45–54.
6 Zhu BCR, Laine RA (1996) *Glycobiology* **6:** 811–16.
7 White T, Bennett EP, Takio K, Sorensen T, Bonding N, and Clausen H (1995) *J. Biol. Chem.* **270:** 24156–65
8 Kościelak J, Pacuszka T, Kubin J, Zdziechowska H (1987) *Glycoconj. J.* **4:** 43–9.
9 Antoniewicz J, Bykowska K, Zdebska E, Kościelak J (1989) *FEBS Lett.* **244:** 388–90.
10 Kościelak J, Antoniewicz-Papis J, Zdebska E, Maj S, Leszko B (1995) *Acta Biochim. Polon.* **42:** 35–40.
11 Kamińska J, Glick MC, Kościelak J (1998) *Glycoconj. J.* **15:** 783–8.
12 Longmore GD, Schachter H (1982) *Carbohydrate Res.* **100:** 365–392.
13 Chester MA, Yates AD, Watkins WM (1976) *Eur. J. Biochem.* **69:** 583–92.
14 Zieleński J, Kościelak J (1982) *Eur. J. Biochem.* **125:** 323–9.
15 Zieleński J, Kościelak J (1983) *FEBS Lett.* **158:** 164–8.
16 Voynow JA, Scanlin TF, Glick MC (1988) *Anal. Biochem.* **168:** 367–73.
17 Voynow JA, Kaiser RS, Scanlin TF, Glick MC (1991) *J. Biol. Chem.* **266:** 21572–77.
18 Laemmli UK (1970) *Nature* **227:** 680–5.
19 Segel IH (1975) *Enzyme kinetics. Behavior and analysis of rapid equilibrium of steady state enzyme systems*. Wiley and Sons, Inc., New York, London, Sydney, Toronto.
20 Szewczyk A, Poddana H, Nalęcz MJ (1990) *Acta Biochim. Polon.* **37:** 113–6.

Received 30 November 1999; revised 4 February 2000; accepted 10 February 2000