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RESEARCH ARTICLE

Dyes with High Affinity for Diphtheria Toxoid as Promising Affinity Ligands for its Industrial Purification

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Abstract: ***Background*:** Usually, diphteria toxin concentration and purification are performed after detoxification due to its high danger. In this work, an alternative diphteria toxoid (DTx) concentration and purification method by affinity chromatography is proposed.

***Methods*:** Screening of 19 triazinic dyes for selecting the best ligand for DTx concentration and purification by affinity chromatography was performed.

***Results*:** Cibacron Blue 3GA and Reactive Green 10 showed the highest affinity for DTx. The adsorption isotherms of DTx on Cibacron Blue 3GA-Sepharose and Reactive Green 19-Sepharose showed a good fit of experimental data to a Langmuir-type isotherm and allowed the calculation of a maximum capacity (qm) of 1.21 ± 0.12 µmol DTx/mL matrix and 1.44 ± 0.14 µmol DTx/mL matrix, respectively and a dissociation constant (Kd) of 9.75 ± 2.90 µM and 1.58 ± 0.38 µM, respectively. Blue R-HE-Sepharose isotherm did not show a good fit of experimental data to a Langmuir-type isotherm due to its high Kd.

***Conclusion*:** Cibacron Blue 3GA and Reactive Green 10 are promising ligands for concentration and purification of DTx by affinity chromatography.

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1. INTRODUCTION

Diphtheria is an acute epidemic respiratory and cutaneous disease caused by diphtheria toxin (DT) secreted by *Corynebacterium diphtheriae*. DT is a NAD-dependent enzyme which transfers the ADP-ribose moiety of the dinucleotide to elongation factor 2 (EF2) thus inhibiting eukaryotic protein synthesis [1, 2]. Diphtheria is treated applying anti-diphtheria hyperimmune equine serum [3] and its prevention is attained through an effective vaccination program [4]. Diphtheria toxoid (DTx), the formaldehyde-inactivated toxin, is used as an active immunizing agent against diphtheria in vaccines [5]. In general, for vaccine preparation, DTx partially purified by ultrafiltration is used [6]. Vaccine collateral reactions are related to contaminants coming from formaldehyde reaction with other culture media compounds and other bacterial metabolic products. Adverse reactions to routine vaccines are obstacles to the mass vaccination campaigns. The increase in DTx purity reduces vaccine adverse reactions and allows an increase in antigen doses which enhance protection [7].

There are many protocols to obtain high purity DT and DTx [8-15], in few of them, DT is inactivated at the end of the purification process and so a qualified staff, and a high biosecurity level are needed to manipulate it. In other protocols, DT is inactivated at the beginning of the process and therefore it can be manipulated using less biosecurity level facilities and less qualified staff. Most of the protocols described to obtain high purity DT and DTx are too expensive for their application in industrial vaccine production. These protocols consist in multiple separation steps including precipitation and several chromatography steps like hydrophobic interaction chromatography, ionic exchange chromatography, size exclusion chromatography and affinity chromatography.

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Affinity chromatography is ideally suited for the purification of therapeutic proteins as it is the most effective method for direct isolation and purification of biomolecules from complex mixtures. Its good selectivity minimizes contamination and yields products of high purity in a single step [5, 16, 17]. Monoclonal Antibodies (mAbs) display high affinity and selectivity but are expensive and can lose activity or leach into products by the harsh elution and cleaning conditions used in industrial purification processes. On the other hand, triazine dyes have been widely used to obtain pseudobiospecific ligand affinity chromatography matrices due to their low cost, availability, simple immobilization reaction, biological and chemical degradation resistance, and acceptable selectivity and capacity [18, 19]. Some dyes, such as Cibacron Blue and Procion Red HE-3 have been used for a variety of NAD+ and NADP+ dependent enzymes purification as well as a wider range of proteins [20]. Particularly, Cibacron Blue 3GA has been proposed for DT purification [10]. Protein adsorption to a triazine dye is very complex and depends on electrostatic and/or hydrophobic interactions, van der Walls forces and hydrogen bridges. Therefore, the interaction between a protein and a certain dye is very difficult to predict [19].

Although Cibacron Blue has been proposed to purify DT [10], there are no previous studies on dyes with affinity to DTx. A dye with affinity to DTx could be useful for its concentration and purification, which could allow DT inactivation at the beginning of the purification process, thus avoiding the risk of manipulating DT.

The aim of this work was to find dyes with high affinity for DTx, potentially useful for its purification by dye affinity chromatography.

2. MATERIALS AND METHODS

2.1. Reagents and Materials

Pre-swollen in 20% ethanol Sepharose 6 FF (Code number: 17-0159-0) was from GE Healthcare (Waukesha, WI, U.S.A.). Reactive Black 5, Reactive Brown 10, Reactive Yellow 2, Reactive Yellow 86, Reactive Blue 4, Reactive Blue 5, Remazol Brilliant Violet 5R, Reactive Blue 15, Reactive Red 4, Reactive Green 19 and Cibacron Blue 3GA dyes were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Rojo Vilmafix 7B-HE, Escarlata Vilmafix G-A and Rojo Vilmafix 3B-HE (Red-120) were from Vilmax S. A. (Buenos Aires, Argentina). Red F5B (Reactive Red 2), Yellow FR, Orange R-HE, Blue R-HE, Yellow 4R-HE were kindly provided by Multicrom S.A.I.C. (Buenos Aires, Argentina). Purified diphtheria toxoid was from INPB ANLIS-Malbrán (Buenos Aires, Argentina). Bio-Rad Protein Assay Dye Reagent was from Bio-Rad Laboratories (Philadelphia, PA, U.S.A.). All other reagents were AR grade.

2.2. Methods

2.2.1. Dye-Sepharose 6 FF Preparation

Each reactive dye was immobilized on 1 mL pre-swollen in 20% ethanol Sepharose 6 FF, using an adapted protocol from Stellwagen [21]. Sepharose 6 FF was washed with five volumes of H_2O. Each dye (50 mg) was dissolved in 1 mL H_2O and added to each Sepharose portion. Then, 1 mL 2 N NaCl was added. To the vessels containing cold dyes (Reactive Brown 10, Reactive Blue 4, Escarlata Vilmafix G-A, Red F5B and Yellow FR), 0.5 mL 0.1 N NaOH was added, and to the vessels containing hot dyes (Reactive Black 5, Reactive Yellow 2, Reactive Yellow 86, Reactive Blue 5, Remazol Brilliant Violet 5R, Reactive Blue 15, Reactive Red 4, Reactive Green 19 and Cibacron Blue 3GA, Rojo Vilmafix 7B-HE, Rojo Vilmafix 3B-HE, Orange R-HE, Blue R-HE and Yellow 4R-HE) 0.5 mL 1 N NaOH was added. All vessels were stirred for 72 h at room temperature. Then, the matrices were washed sequentially with 5 mL H_2O, 1 M NaCl, 2M ammonium sulphate and H_2O. The matrices were stored in 2 M NaCl.

2.2.2. Protein Concentration Measurement

Protein concentration was measured according to Bradford [22].

2.2.3. Screening Experiments: DTx Adsorption on Dye-Sepharose 6 FF Columns

Nineteen columns (0.5 × 5 cm) were prepared, each having one of the different dye-Sepharose matrices and they were equilibrated with the starting buffer. Pure samples of DTx (8.35 mg/mL) in the same buffer were loaded on each column. The columns were then washed with equilibrating buffer at a flow rate of 0.25 mL/min until the absorbance at 280 nm reached its initial value. When the equilibrating buffer was phosphate buffered saline (PBS) pH 7.4, elution was performed with 1 M KCl in the equilibrating buffer, while when the equilibrating buffer was 20 mM sodium citrate, pH 3.0, 150 mM NaCl, the elution was accomplished with 20 mM sodium phosphate buffer, pH 7.0, at the same flow rate. Different NaCl concentrations (150 mM, 200 mM and 250 mM) in the citrate buffer were also assessed with those columns showing the best performance (Blue R-HE, Reactive Green 19 and Cibacron Blue 3GA-Sepharose).

2.2.4. DTx Adsorption Isotherm Determination

Three hundred μL of pre-swollen Dye-Sepharose 6 FF matrixes were placed in graduated tubes. Then 10 mL of pure DTx solution at different concentrations in 20 mM sodium citrate buffer, pH 3.0, 200 mM NaCl, was added. The suspension was gently shaken overnight at 24ºC to enable the system to reach its equilibrium. Protein concentration was determined with Bradford reagent. The equilibrium concentration of DTx bound to the matrix was calculated as the total amount of DTx present at the beginning of the experiment less the amount still in the soluble phase at equilibrium. The qm and Kd values were calculated using a one-to-one Langmuir binding model as described by Chase [23] using the SigmaPlot 2001 regression program (2001 SPSS Inc.).

3. RESULTS AND DISCUSSION

3.1. DTx Adsorption to Dye-Sepharose Matrices

Table **1** shows the percentage of adsorption of DTx on the 19 matrices with different buffers. When working with

Table 1. Diphtheria toxoid adsorption on the 19 matrices synthesized.

Dye/Buffer	Adsorption Percentage			
	20 mM Sodium Phosphate Saline, pH 7.0 (PBS)	20 mM Sodium Citrate, 150 mM NaCl, pH 3.0	20 mM Sodium Citrate, 200 mM NaCl, pH 3.0	20 mM Sodium Citrate, 250 mM NaCl, pH 3.0
REACTIVE BLACK 5*	3	-	-	-
REACTIVE BROWN 10*	5	-	-	-
ROJO VILMAFIX 7B-HE*	9	-	-	-
REACTIVE YELLOW 2*	19	-	-	-
REACTIVE YELLOW 86*	29	-	-	-
REACTIVE BLUE 5*	ND	-	-	-
REACTIVE BLUE 4	ND	40	-	-
REMAZOL BRILLIANT VIOLET 5R	ND	33	-	-
REACTIVE BLUE 15	7	49	-	-
ESCARLATA VILMAFIX G-A	2	30	-	-
ROJO F5B (REACTIVE RED 2)*	4	-	-	-
YELLOW FR*	8	-	-	-
REACTIVE RED 4*	ND	-	-	-
ORANGE R-HE	ND	51	-	-
BLUE R-HE	24	75	86	85
YELLOW 4R-HE*	14	-	-	-
REACTIVE GREEN 19	14	71	86	78
ROJO VILMAFIX 3B-HE (RED-120)*	12	-	-	-
CIBACRON BLUE 3GA	8	78	94	89

* These matrices were discarded as they presented low adsorption percentages (< 10%) at pH 3.0.

PBS, all matrices showed a low adsorption percentage. When employing 20 mM sodium citrate, pH 3.0, 150 mM NaCl, as the adsorption buffer, Blue R-HE, Reactive Green 19 and Cibacron Blue 3GA showed the best results, adsorbing more than 71% of the DTx loaded. The columns with Sepharose with Reactive Black 5, Reactive Brown 10, Rojo Vilmafix 7B-HE, Reactive Yellow 2, Reactive Yellow 86, Reactive Blue 5, Red F5B (Reactive Red 2), Yellow FR, Reactive Red 4, Yellow 4R-HE, Rojo Vilmafix 3B-HE (Red 120) immobilized, presented a low percentage of adsorption, therefore they were discarded for further experiments.

DTx adsorption to Blue R-HE, Reactive Green 19 and Cibacron Blue 3GA Sepharose were assessed employing 20 mM sodium citrate, pH 3.0, with NaCl at different concentrations as the adsorption buffer. When NaCl concentration was increased from 150 to 200 mM, DTx adsorption improved significantly, while higher NaCl concentrations did not modified significantly the DTx adsorption percentage. Therefore, 20 mM sodium citrate buffer, pH 3.0, 200 mM NaCl, was chosen as the adsorption buffer for further experiments.

3.2. Adsorption Isotherms at pH 3.0

Fig. (**1**) shows the adsorption isotherms of DTx on Blue R-HE, Reactive Green 19 and Cibacron Blue 3GA-Sepharose, developed with pure DTx in 20 mM sodium citrate buffer, pH 3.0, 200 mM NaCl. For Cibacron Blue 3GA-Sepharose and Reactive Green 19, the isotherms showed a good fit of experimental data to a Langmuir-type isotherm and allowed the calculation of a maximum capacity (qm) of 1.21 ± 0.12 μmol DTx/mL matrix and 1.44 ± 0.14 μmol DTx/mL matrix respectively, and a Kd of 9.75 ± 2.90 μM and 1.58 ± 0.38 μM respectively. Blue R-HE-Sepharose isotherm did not show a good fit of experimental data to a Langmuir-type isotherm and show a high Kd.

Taking into account that DT is a NAD-dependent enzyme, Fratelli *et al.* [11] has previously proposed a method to purify DT by affinity chromatography with Cibacron Blue F3GA equilibrated with PBS and eluting the DT using a linear KCl gradient (0–1 M) in the same buffer. This type of interaction occurs because this dye mimics the structure of NAD. However, as it is well known, working with DT needs

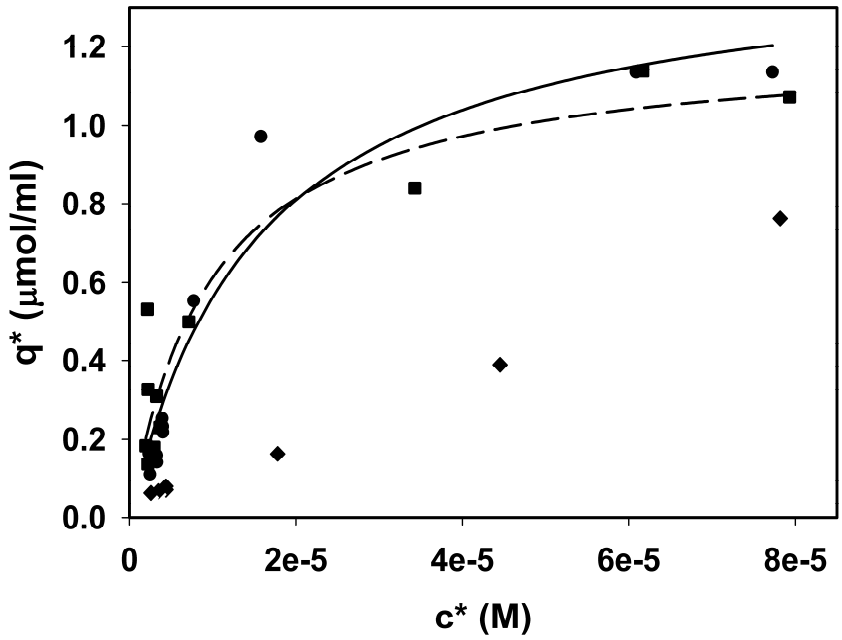

Fig. (1). Equilibrium adsorption isotherms.
Equilibrium adsorption isotherms for binding of DTx to Blue R-HE –Sepharose (◆), Reactive Green 19-Sepharose (●) and Cibacron Blue 3GA-Sepharose (■). Matrix aliquots, 300 µL, were put into tubes containing 10 mL of pure DTx solution at different concentrations in 20 mM sodium citrate buffer, 200 mM NaCl, pH 3.0. The suspension was gently shaken overnight at 24°C and protein concentration in the supernatant was measured with Bradford reagent. Kd of 9.75 ± 2.90 µM and 1.58 ± 0.38 µM for Cibacron Blue 3GA-Sepharose and Reactive Green 19-Sepharose, respectively, and qm of 1.21 ± 0.12 and 1.44 ± 0.14 µM DTx for Cibacron Blue 3GA -Sepharose and Reactive Green 19-Sepharose, respectively, were calculated. Blue R-HE-Sepharose isotherm did not showed a good fit of experimental data to a Langmuir-type isotherm.
(----) Isotherm of Cibacron Blue 3GA-Sepharose.
(—) Isotherm of Reactive Green 19-Sepharose.

a high biosecurity level due to its high toxicity, being more convenient to inactivate DT and then purify DTx.

In this work, we studied the adsorption of DTx to nineteen Dye-Sepharose columns, including Cibacron Blue 3GA Sepharose, equilibrated with PBS. In that condition, all matrices showed a low adsorption percentage, thus making this buffer unsuitable for DTx purification. Probably, formaldehyde converts DT into DTx by destroying active sites in the molecule, such as the NAD+ binding cavity and the receptor-binding site. Carroll *et al.* [24], previously found that nucleotide-free toxoid was unable to bind to columns containing triazinyl ligands mimicking NAD+. We evaluated other adsorption conditions because it is known that dyes not only bind to NAD+ and NADP+ dependent enzymes but also to a wide range of proteins by a combination of electrostatic and/or hydrophobic interactions, van der Walls forces and hydrogen bridges [19, 25]. Most of the studied matrices adsorbed different percentages of DTx when working with sodium citrate at pH 3.0 as the adsorption buffer. Blue R-HE, Reactive Green 19 and Cibacron Blue 3GA showed the best results. The adsorption isotherms showed that Reactive Green 19 and Cibacron Blue 3GA adsorbed DTx with higher affinity than Blue R-HE. The qm with Reactive Green 10 and Cibacron Blue 3GA were comparable to commercial matrices [8]. The moderate Kd values are just in the range of desired affinity interaction applicable to affinity chromatography separation of proteins (10^5-10^6 L/mol) [26] allowing the DTx adsorption at low concentrations from culture supernatants and then its elution under mild conditions without impairing its biological activity. According to their qm and Kd values, Reactive Green 10 seems to be more suitable than Cibacron Blue 3GA as the ligand for DTx purification by affinity chromatography.

CONCLUSION

The low cost of the selected dye ligands and their adequate affinity for DTx make them promising ligands for the industrial scale-up of the chromatographic processes for its purification by affinity chromatography.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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MCMC, SAC and OC designed the experiments. MCMC and SLG performed the experiments. MCMC, SAC, OC and SLG analyzed the results. OC and JCD provide pure DTx. All authors were involved in manuscript writing.

REFERENCES

[1] Honjo, T.; Nishizuka, Y.; Hayaishi, O. Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem.,* **1968**, *243*, 3553-3555.

[2] Gill, D.M.; Pappenheimer, A.M., Jr.; Brown, R.; Kurnick, J.T. Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. *J. Exp. Med.,* **1969**, *129*, 1-21.

[3] Funke, G.; Von Graevenitz, A.; Clarridge, J., 3rd; Bernard, K. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.,* **1997**, *10*, 125-159.

[4] Rappuoli, R.; Pizza, M.; Del Giudice, G.; De Gregorio, E. Vaccines, new opportunities for a new society. *Proc. Natl. Acad. Sci. U.S.A.,* **2014**, *111*, 12288-12293.

[5] Brooks, G.F.; Bennett, J.V.; Feldman, R.A. Diphtheria in the United States. 1959-1970. *J. Infect. Dis.,* **1974**, *129*, 172-178.

[6] Rappuoli, R. In: *New generation vaccines;* Woodrow, G.C.; Levine, M.M., Ed.; Marcel Dekker: New York, **1990**; pp. 251-268.

[7] Relyveld, E.H.; Bizzini, B.; Gupta, R.K. Rational approaches to reduce adverse reactions in man to vaccines containing tetanus and diphtheria toxoids. *Vaccine,* **1998**, *16*, 1016-1023.

[8] Carroll, S.F.; Barbieri, J.T.; John Collier, R. In: *Methods Enzymo*l: *Microbial Toxins: Tools in Enzymology.* 1st ed., Sidney, H., Ed.; Academic Press: La Jolla, **1988**, Vol. *165*, pp. 68-76.

[9] Eaton, M.D. The purification and concentration of diphtheria toxin: II. Observations on the nature of the toxin. *J. Bacteriol.,* **1936**, *31*, 367-383.

[10] Antoni, G.; Bigio, M.; Borri, G.; Casagli, M.C.; Neri, P. Purification of diphtheria toxin by chromatography on Cibacron Blue-Sepharose. *Experientia,* **1983**, *39*, 885-886.

[11] Fratelli, F.; Abrahão-Neto, J.; Prestia Caricati, A.T.; Marques Borges, M.; Guidolin, R.; Caricati, C.P. An alternative method for purifying and detoxifying diphtheria toxin. *Toxicon,* **2011**, *57*, 1093-100.

[12] Cox, J.C. New method for the large-scale preparation of diphtheria toxoid: purification of toxin. *Appl. Microbiol.,* **1975**, *29*, 464-468.

[13] Cukor, G.; Readio, J.D.; Kuchler, R.J. Affinity chromatography purification of diphtheria toxin. *Biotechnol. Bioeng.,* **1974**, *16*, 925-931.

[14] Nitzan, Y.; Michalsky, T. Simple procedure for purification of diptheria toxin and separation of its fragments by hydrophobic chromatography. *Anal. Biochem.,* **1980**, *109*, 71-75.

[15] Goerke, A.R.; Svab, T.; Mchugh, P.; Valente, K. 2014. Methods of purification of native or mutant forms of diphtheria toxin. WO Patent 2012173876A1, Dic, 20, 2012.

[16] Sproule, K.; Morrill, P.; Pearson, J.C.; Burton, S.J.; Hejnaes, K.R.; Valore, H.; Ludvigsen, S.; Lowe, C.R. New strategy for the design of ligands for the purification of pharmaceutical proteins by affinity chromatography. *J. Chromatogr. B Biomed. Sci. Appl.,* **2000**, *740*, 17-33.

[17] Roque, A.C.; Lowe, C.R. In *Affinity chromatography: methods and protocols*; Zachariou, M., Ed.; Humana Press, Totowa, **2007**; Vol. *421*, pp. 1-21.

[18] Lowe, C.R.; Pearson, J.C. In: *Methods Enzymol: Enzyme Purification and Related Techniques, Part C,* William, B.J., Ed.; Academic Press: London, **1984**, Vol. *104*, pp. 97-113.

[19] Scopes, R.K. Strategies for enzyme isolation using dye-ligand and related adsorbents. *J. Chromatogr. B: Biomed. Sci. Appl.,* **1986**, *376,* 131-140.

[20] Batista-Viera, F.; Janson, J.C.; Carlsson, J. In: *Protein Purification: Principles, High-Resolution Methods and Applications*; Janson, J.C. Ed.; John Wiley & Sons, Inc. Hoboken, **2011**, pp. 221-258.

[21] Stellwagen, E. In: Methods Enzymol: Guide to Protein Purification; Deutschler, M.P., Ed.; Academic Press, London, **1990**; Vol. *182*, pp. 343-57.

[22] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **1976**, *72*, 248-54.

[23] Chase, H.A. Prediction of the performance of preparative affinity chromatography. *J. Chromatogr.,* **1984**, *297*, 179-202.

[24] Carroll, S.F.; Barbieri, J.T.; Collier, R.J. Dimeric form of diphtheria toxin: purification and characterization. *Biochemistry,* **1986**, *25*, 2425-30.

[25] Metz, B. *Structural Characterisation of Diphtheria Toxoid.* PhD Thesis, Utrecht University, Utrecht, Holanda, **2005**.

[26] Hermanson, G.T.; Mallia, A.K.; Smith, P.K. In: *Immobilized affinity ligand techniques*; Hermanson, G.T.; Mallia, A.K.; Smith, P.K., Ed.; Academic Press; San Diego, **1992**, pp. 137-280.