0273-2289/03/104/0173/$12.75

Purification of Neutral Protease by Dye Affinity Chromatography

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Received May 1, 2001; Revised June 1, 2002; Accepted July 1, 2002

Abstract

Twenty triazinic dyes were assayed as ligands for the chromatographic affinity purification of a neutral protease from Flavourzyme™, a commercial preparation. Screening at pH 4.0 allowed the selection of eight dyes on the basis of their high protease adsorption. When the pH was set to 5.0 in order to increase selectivity, only Yellow HE-4R, Red HE-3B, and Cibacron Blue F3G-A maintained protease adsorption at high values. Neither maximum capacities nor dissociation constants calculated from isotherms measured at 8 and 25°C showed great differences. By contrast, a strong temperature effect was evidenced in the elution step: elution at 8°C allowed 70, 81, and 98% recovery of adsorbed protease with Yellow HE-4R, Red HE-3B, and Cibacron Blue F3G-A, respectively, whereas only 20% recovery was attained at 25°C. Based on the results obtained, a purification process for the neutral protease contained in Flavourzyme with Cibacron Blue F3G-A as the affinity ligand was developed, yielding 96% of electrophoretically pure enzyme in a single step, the specific activity rising from 850 to 3650 U/mg.

Index Entries: Enzymes; neutral protease; purification; dye affinity chromatography; temperature effect.

Introduction

Triazine dyes have been extensively utilized to obtain affinity chromatography matrices owing to their low cost, ready availability, simple immobilization reaction, biological and chemical degradation resistance,

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and acceptable capacity *(1)*. All these advantages make them highly eligible for large-scale protein purification *(2)*.

A biospecific interaction between a natural ligand-binding site and a dye involves only a few dyes, and the strength of the interactions is found to vary from dye to dye. To find an appropriate selective interaction, it is necessary to screen a range of adsorbents, possibly under more than one buffer condition *(3)*.

In early studies, reactive dyes were used almost exclusively to purify dehydrogenases and kinases because of their affinity for these enzymes. Reactive dyes—as NAD analogs—interact specifically with dehydrogenases and kinases. In addition, because reactive dyes contain polar and nonpolar groups, they can interact rather selectively with other proteins by both hydrophobic and electrostatic forces. Elution conditions may be selective in the case of enzymes, especially those interacting with nucleotides or phosphorylated substrates, or nonselective *(4)*.

Some factors affecting the adsorption and elution steps have been extensively studied (e.g., pH, ionic strength). However, few studies have examined the effects of adsorption and desorption temperature in group-specific affinity processes, and all of them deal only with dehydrogenases or kinases. Temperature can affect protein adsorption on immobilized dyes, and like pH and ionic strength, these effects vary from case to case, depending on the types of interactions involved *(5)*. Harvey et al. *(6)* reported that the higher the temperature the lower the affinity of glycerokinase and yeast alcohol dehydrogenase for 5'-AMP-Sepharose, suggesting that linear gradients of temperature can be successfully employed for the elution of these enzymes. With other proteins, the effect of temperature on adsorption is less predictable.

Chromatography on immobilized dyes has rarely been used in protease purification *(7)*. In several cases, the enzyme purification process involves a combination of sequential ion-exchange, dye-ligand, and gel filtration chromatography *(8–10)*.

In the present study, we examined the purification of the neutral protease contained in a commercial preparation by using triazine dyes as the affinity ligands, and assessing the effect of temperature on the adsorption and elution steps for the purpose of developing an economical, simple, and easy-to-scale-up purification process.

Materials and Methods

Reagents and Dyes

Flavourzyme™ (Novo Industries, Denmark) was used as the source of neutral protease. Sepharose CL-4B was from Amersham, Uppsala, Sweden. The origins of the triazinic dyes are given in Table 1. Azocasein was from Sigma-Aldrich, St. Louis, MO. All other reagents were of analytical grade.

Table 1
Triazine Dyes Immobilized on Sepharose CL-4B

Dye name	Type[a]	Dye density (μmol/g)	Supplier[b]
Reactive Black 5	A	3.8	Sigma
Reactive Brown 10	D	3.2	Sigma
Red 7B-HE	M	3.3	Vilmax
Reactive Yellow 2	M	3.9	Sigma
Reactive Yellow 86	M	3.8	Sigma
Reactive Blue 5	M	3.9	Sigma
Reactive Blue 4	D	3.3	Sigma
Remazol Brilliant Violet 5R	A	3.1	Sigma
Reactive Blue 15	M	3.3	Sigma
Scarlet Vilmafix G-A	D	3.7	Vilmax
Red F5B	D	3.8	Multicrom
Yellow FR	D	3.8	Multicrom
Reactive Red 4	M	3.3	Sigma
Orange R-HE	M	3.6	Multicrom
Blue R-HE	M	3.7	Multicrom
Yellow HE-4R	M	3.4	Multicrom
Reactive Green 19	M	3.1	Sigma
Reactive Green 5	M	3.2	Sigma
Red HE-3B	M	3.8	Vilmax
Cibacron Blue F3G-A	M	3.7	Sigma

[a]M, monochlorotriazine; D, dichlorotriazine; A, achlorotriazine.
[b]Sigma-Aldrich, St. Louis, MO; Vilmax S.A., Buenos Aires, Argentina; Multicrom S.R.L., Buenos Aires, Argentina.

Preparation of Dye–Sepharose 4B

Reactive dyes were immobilized on Sepharose CL-4B as described by Stellwagen *(11)* with minor modifications: Ten grams of Sepharose CL-4B was suspended in 20 mL of water and 500 mg of dye was added prior to the addition of 10 mL of 2 *M* NaCl. Then, 5 mL of 0.1 *N* NaOH was added in the case of a dichlorotriazine dye being used, and the mixture was gently stirred for 4 h at room temperature. If a monochlorotriazine or achlorotriazine dye was utilized, 5 mL of 1 *N* NaOH was added and the mixture stirred for 16 h at 60°C. The mixture was then filtered and the matrix washed with water, 1 *M* NaCl, 2 *M* ammonium chloride, and water once again, until the filtrate was clear. The immobilized dyes are given in Table 1.

The amount of immobilized dye was determined by acid hydrolysis followed by a spectrophotometric measurement as per Burton et al. *(12)*. The immobilized dye concentration is expressed as micromoles of dye per gram wet weight of dye-Sepharose (Table 1).

Protease Purification Screening Experiments

One-milliliter bed volume (1 × 1.3 cm) columns were prepared, each having one of the 20 different dye-Sepharose matrices. Each column was

then equilibrated with 12 mL of 20 m*M* sodium acetate buffer at the preset pH, and 0.5 mL of 5 mg/mL Flavourzyme in the same buffer was applied. After a washing step with 5 mL of equilibrating buffer, elution was performed with 4 mL of 1 *M* NaCl in equilibrating buffer. Flow rate was 0.25 mL/min. Samples from washing and elution steps were desalted by passing them through a PD-10 column and analyzed for proteolytic activity by the azocasein method *(13)*. Columns were regenerated by flushing with 6 mL of 0.05 *N* NaOH followed by 6 mL of water.

Proteolytic Activity Assay

The proteolytic activity assay was carried out by the method of Charney and Tomarelli *(14)* as modified by Vázquez et al. *(13)*. One protease unit is defined as the amount of enzyme that causes a rise in absorbance at 340 nm of 0.003/min, at 55°C and pH 6.0.

Measurement of Protein Concentration

Protein concentration was measured as described by Bradford *(15)*.

Electrophoresis

A 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Hoefer SE200 Mighty Small minigel unit (Pharmacia Biotech, Uppsala, Sweden) using the discontinuous method described by Laemmli *(16)*.

Isotherm Measurement

The adsorption isotherms for protease binding to dye matrices were measured in batch systems, basically as described by Chase *(17)*, with a 20 m*M* sodium acetate buffer, pH 4.0, as the adsorption buffer. Matrices (50 µL) were put into tubes containing 1–18 mg of Flavourzyme in the adsorption buffer, in a final volume of 1 mL. The suspension was stirred gently for 24 h to allow the system to reach equilibrium. The supernatant was then removed and protease concentration at equilibrium (c^*) was determined. The equilibrium concentration of protease bound to the matrix per unit of matrix volume (q^*) was calculated as the total amount of enzyme present at the beginning of the experiment minus the amount still in the soluble phase at equilibrium. Maximum binding capacity and dissociation constant were calculated from the isotherm as per Chase *(17)*.

Results and Discussion

Purification Screening Experiments

A 20 m*M* sodium acetate buffer, pH 4.0, was used as the adsorption buffer, taking into account that proteins tend to bind to triazine dyes more tightly at lower pH values, presumably because of a contribution from ionic interactions favored by increasing positive charges on protein molecules *(18)*.

Table 2
Screening of Dye-Sepharose Chromatographic Matrices for Protease Adsorption at Different pH Values

	Adsorbed protease (%)	
Dye	pH 4.0	pH 5.0
Reactive Black 5	63	
Reactive Brown 10	86	
Red 7B-HE	92	
Reactive Yellow 2	87	
Reactive Yellow 86	50	
Reactive Blue 5	93	
Reactive Blue 4	45	
Remazol Brilliant Violet 5R	84	
Reactive Blue 15	85	
Scarlet Vilmafix G-A	46	
Reactive Red 2	94	
Yellow FR	97	60
Reactive Red 4	98	46
Orange R-HE	97	68
Blue R-HE	98	66
Yellow HE-4R	97	84
Reactive Green 19	94	
Reactive Green 5	98	67
Red HE-3B	98	87
Cibacron Blue F3G-A	99	82

A 20 m*M* sodium acetate buffer, pH 4.0, 1 *M* NaCl was utilized to elute adsorbed proteins on dye columns because it provides a useful elution condition not so hard as to cause protein denaturation. Hydrophobic eluents failed to elute significant amounts of protease (see Eluent Selection).

Table 2 gives the results of the protease purification screening. Eight of the 20 dyes assayed showed a retained fraction of >95% at pH 4.0. When, in order to increase the selectivity, pH was raised to 5.0, only Cibacron Blue F3G-A, Red HE-3B, and Yellow HE-4R conserved the retained fraction >80%. At pH >5.5, no protease was adsorbed by any of these three dyes.

Taking into account the results of the screening, we selected Cibacron Blue F3G-A, Red HE-3B, and Yellow HE-4R for further experiments.

A control column with S-Sepharose instead of dye-Sepharose, at pH 4.0, was only able to retain 25% of the protease, thus indicating that the immobilized dye was acting as affinity ligand rather than as cation exchanger.

Selection of Eluent

After saturation of the dye matrices with Flavourzyme at pH 4.0, different nonselective eluents were assayed. Table 3 displays the results obtained, showing that 1 *M* NaCl in 20 m*M* sodium acetate buffer, pH 4.0, was the most effective eluent for the adsorbed protease on the three dye

Table 3
Eluent Selection for Protease Adsorbed on Selected Dye-Sepharose Columns[a]

Dye	1 *M* NaCl	50% Ethyleneglycol	50% Ethyleneglycol, 1 *M* NaCl	25% Isopropanol	25% Isopropanol, 1 *M* NaCl
Yellow HE-4R	70	2	52	3	48
Red HE-3B	81	1	68	2	50
Cibacron Blue F3G-A	98	1	61	2	56

[a]Results are expressed as percentage of elution.

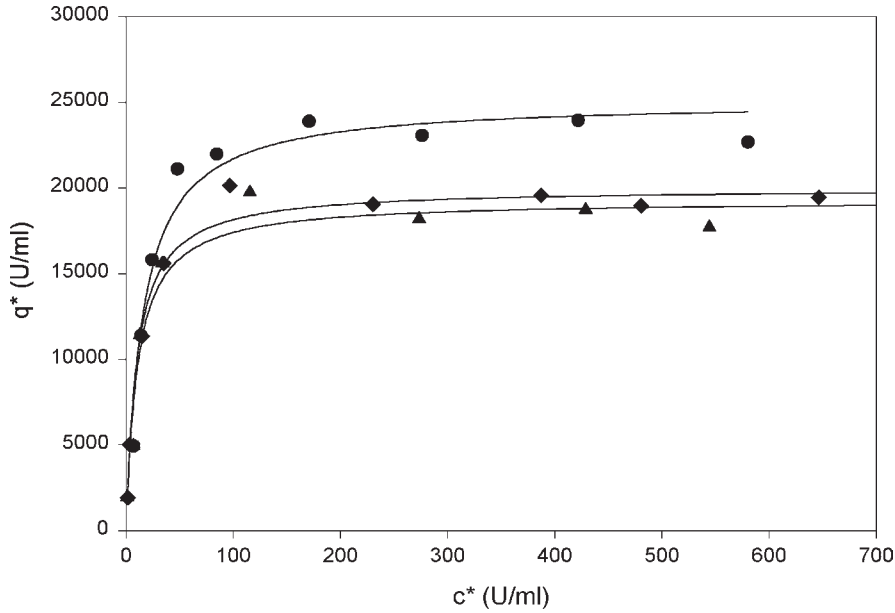

Fig. 1. Adsorption isotherms of neutral protease from Flavourzyme on Yellow HE-4R– (●), Red HE-3B– (▲), and Cibacron Blue F3G-A (◆)–Sepharose matrices, at 8°C. See Materials and Methods for details.

matrices, thus providing evidence that binding forces are mainly electrostatic. When the pH of the eluent was raised to 5.0, no significant improvement in protease elution was attained.

Influence of Temperature on Chromatographic Behavior of Neutral Protease

Since temperature can have some influence on the adsorption and/or elution steps, its influence was assessed in two separate experiments: that on the adsorption step was assessed by developing the isotherms at two different temperatures (8 and 25°C) while its bearing on the elution step was assessed by performing the whole operation at different temperatures.

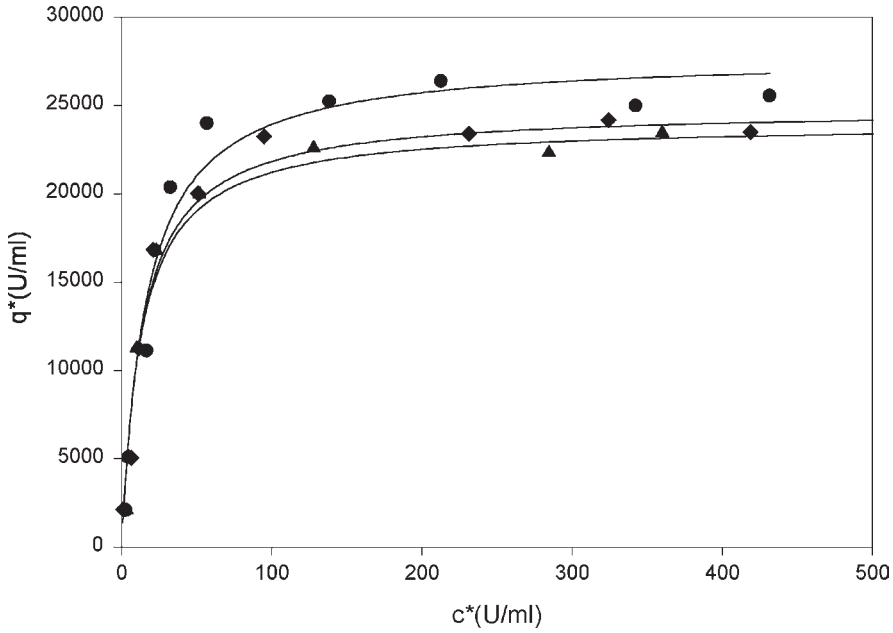

Fig. 2. Adsorption isotherms of neutral protease from Flavourzyme on Yellow HE-4R– (●), Red HE-3B– (▲), and Cibacron Blue F3G-A (◆)–Sepharose matrices, at 25°C. See Materials and Methods for details.

Table 4
Parameters Calculated from Adsorption Isotherms Developed on Selected Dye-Sepharose Matrices at Different Temperatures

	8°C		25°C	
Dye	q_m (U/mL)[a]	K_d (U/mL)[b]	q_m (U/mL)[a]	K_d (U/mL)[b]
Yellow HE-4R	25,080	16	27,810	16
Red HE-3B	19,260	11	23,970	13
Cibacron Blue F3G-A	19,980	10	24,790	14

[a]q_m = maximum capacity.
[b]K_d = dissociation constant.

Adsorption isotherms are shown in Figs. 1 and 2. Maximum capacity and dissociation constants calculated from the isotherms obtained at 8 and 25°C were very similar (Table 4), thus indicating that temperature does not have any significant influence on the adsorption step. Adsorption increased by only 10–15% when the temperature was raised from 8 to 25°C, as judged by the maximum binding capacities obtained from the adsorption isotherms.

After saturation of the dye matrices with Flavourzyme, the influence of temperature on protease elution with 1 *M* NaCl in sodium acetate buffer, pH 4.0, was assessed. Figure 3 shows the results obtained, evidencing the strong effect of temperature on the degree of elution of protease from the

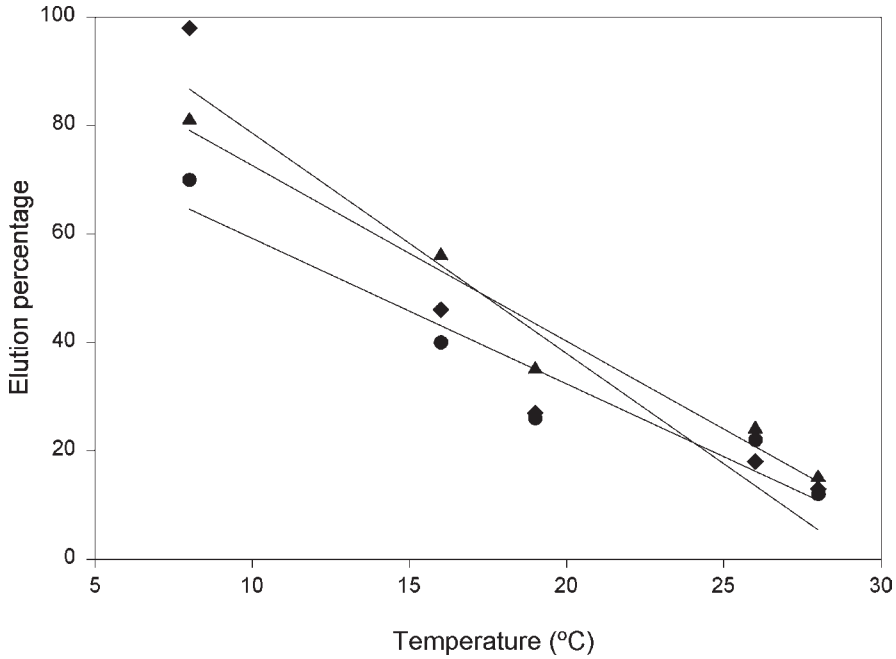

Fig. 3. Effect of temperature on elution of neutral protease from Flavourzyme from Yellow HE-4R– (●), Red HE-3B– (▲), and Cibacron Blue F3G-A (◆)–Sepharose matrices. Columns (1 × 1.3 cm) were saturated with Flavourzyme in a 20 m*M* sodium acetate buffer (pH 4.0) at 8, 16, 19, 26, and 28°C and then eluted with 1 *M* NaCl in the same buffer at the same temperatures.

three dye-affinity matrices selected. Because all three columns had adsorbed approximately the same amount of protease, the great difference in eluted protease can only be ascribed to temperature. With Cibacron Blue F3G-A, almost full elution could be achieved at 8°C but only 20% at 25°C. The effect of temperature on the elution of proteins other than dehydrogenases and kinases from a dye matrix is not predictable, so we advise that this effect be checked because it can be very strong.

We observed that salt promotes the breakdown of the adsorbent–adsorbate complex more efficiently at a low temperature (8°C) than at a higher temperature (25°C). As far as we know, no such enormous effect of temperature on elution of proteins from dye matrices has been reported. Under the same conditions, adsorption and desorption behavior of lysozyme from Red HE-3B–Sepharose and bovine serum albumin from Cibacron Blue F3G-A–Sepharose were not modified significantly when temperature varied from 8 to 25°C.

Harvey et al. *(6)* reported that the strength of interaction—measured as the eluent concentration at the center of the enzyme peak—between AMP-Sepharose and glycerokinase, alcohol dehydrogenase, and lactate dehydrogenase was apparently reduced at higher temperatures. However, the behavior of the three enzymes, in quantitative terms, was very different.

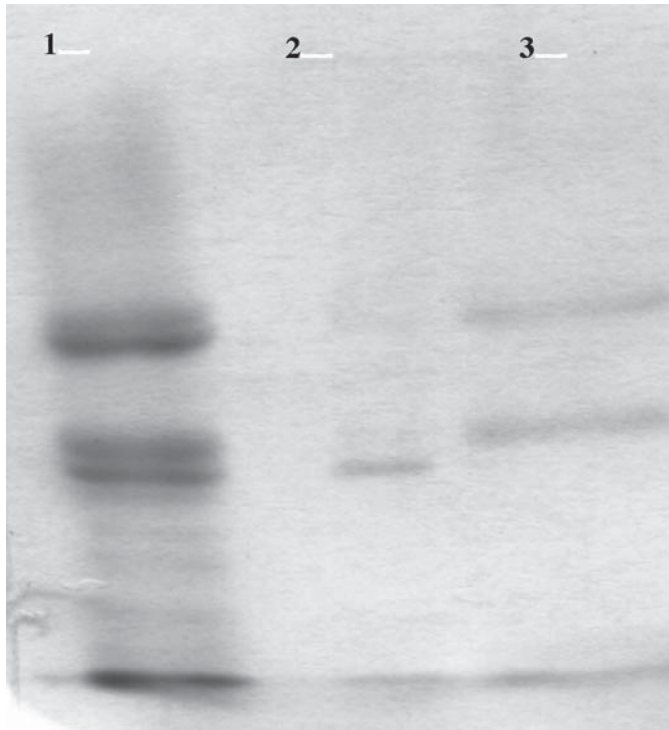

Fig. 4. SDS-PAGE of samples from purification process developed with Cibacron Blue F3G-A as affinity ligand. Lane 1, starting material; lane 2, eluate; lane 3, column washing.

Watson et al. *(19)* purified NAD and NADP-dependent dehydrogenases by using immobilized Cibacron Blue F3G-A and Procion Red HE-3B in experiments conducted at 6°C. No reason, out of enzyme stability, was given for working at that temperature.

The salt-dependent binding of the 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus* has been compared with that of the same enzyme from a mesophile (*Saccharomyces cerevisiae*): the salt concentration required to elute both enzymes from either Cibacron Blue F3G-A or Red HE-3B columns increased while temperature rose up to 45°C. Whereas this effect was observed for thermophilic enzymes on AMP-agarose *(19)*, the opposite was found for mesophilic enzymes *(6)*. Protease from Flavourzyme has an optimum temperature of 50°C, thus demonstrating its thermophilic character.

All these results indicate that temperature can be a very important factor in protein elution from immobilized dyes and should not be overlooked when developing a dye-ligand process. In our case, we optimized the elution step by lowering the temperature from 25 to 8°C, thus increasing the elution capacity from 20 to 70–98%.

Table 5
Purification of Neutral Protease
from Flavourzyme with Cibacron Blue F3G-A–Sepharose

Step	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Starting material	0.40	340	850	100	
Dye-chromatography	0.10	365	3650	91	4.3

Development of Protease Purification Process

Based on the results obtained, purification processes for the neutral protease from Flavourzyme with immobilized Yellow HE-4R, Red HE-3B, and Cibacron Blue F3G-A as affinity ligands were developed. After loading the sample in a 20 m*M* sodium acetate buffer (pH 4.0), and washing until baseline was reached, protease was eluted at 8°C with 1 *M* NaCl in the same buffer. SDS-PAGE (Fig. 4) revealed that while Flavourzyme shows four main bands, two close to 35 kDa (34.5 and 36 kDa) and two close to 50 kDa (50 and 52 kDa), the eluted fraction only shows the band corresponding to the protease (34.5 kDa, revealed by enzyme activity), and the pass-through fraction displays two bands (36 and 52 kDa). The 50-kDa protein remains adsorbed to the matrix, being desorbed in the regeneration step.

A recovery of 70% (Yellow HE-4R), 79% (Red HE-3B), and 96% (Cibacron Blue F3G-A) of essentially pure enzyme was achieved in only one step, demonstrating the usefulness of dye-affinity chromatography for purification of neutral proteases. The purification factor was about 4.3 for the three ligands. Table 5 provides details of the purification process developed with Cibacron Blue F3G-A as the affinity ligand.

Acknowledgments

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica de la República Argentina and the University of Buenos Aires. O.C. is a career researcher of the CONICET.

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