Journal of Chromatography, 376 (1986) 157—161
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2974

AFFINITY PRECIPITATION OF PROTEINS USING BIS-DYES

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SUMMARY

Based on the principle of affinity precipitation of nucleotide-dependent enzymes using bis-NAD (Mosbach), Lowe et al. have recently mentioned the possibility of synthesising bis-dyes for similar applications. In this paper we report preliminary results obtained using bis-dyes in the sulphonamide form got through carbodiimide condensation of the monomer and its aminohexyl derivative for affinity precipitation. The dimer exhibited considerable selectivity for lactate dehydrogenase (90% yield). Bovine serum albumin gave a lower yield of 50% and as expected chymosin could not be precipitated by the dimer.

INTRODUCTION

Fractional precipitation using a range of ammonium sulphate concentrations is a well known step in protein purification. A more selective precipitation, called affinity precipitation, was introduced by Mosbach and Larsson [1] using the dimer of nicotinamide-adenine dinucleotide, oxidized (NAD) for NAD-dependent enzymes such as lactate dehydrogenase (LDH) (EC 1.1.1.27). On the other hand, triazine dyes, used as affinity ligands, are known to imitate the coenzyme and thus exhibit a certain affinity towards the co-factor-dependent enzymes such as LDH [2]. Thus, it is logical to use these dyes as specific bifunctional ligands for affinity precipitation.

In this paper, we present our preliminary results, using a bis-dye, for the purification of LDH.

EXPERIMENTAL

Synthesis of the bis-dye

Commercial Cibacron Blue F3G-A was purified prior to use [3], and its aminohexyl derivative was prepared according to the method described by Small et al. [3]. The dimer was prepared using carbodiimide as the condensing

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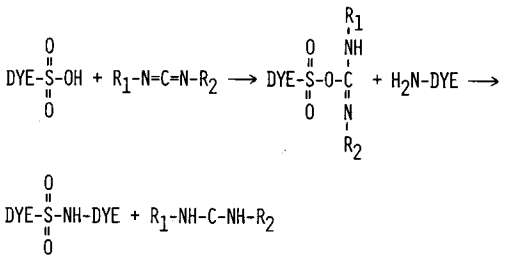

Fig. 1. The hypothetical synthesis of bis-Cibacron Blue.

agent to form the sulphonamide derivative (Fig. 1). The optimal experimental conditions, such as the concentrations of monomer and condensing agent, temperature, etc., were determined.

Precipitation of the different proteins

LDH (EC 1.1.1.27). To 2 ml of the enzymatic solution (1 mg/ml) taken in a test tube, 1 ml of the bis-dye (oligomer) along with 0.5 ml of sodium pyruvate (0.8 *M*) was added and, after slight agitation for one night at 4°C, the mixture was centrifuged at 5000 *g* for 15 min in order to separate the filtrate from the precipitate.

Bovin serum albumin (BSA). BSA was precipitated in a similar manner to LDH but without the addition of sodium pyruvate.

Chymosin (EC 3.4.4.3). Chymosin was precipitated in a similar manner to BSA.

RESULTS

Synthesis of the bis-dye

Table I shows the yield of bis-dye obtained under different concentrations of reagents. A mol/mol ratio of the aminohexyl and underivatized dye with 10 mol of carbodiimide gave the best yield. Qualitative analysis of this preparation using thin-layer chromatography as a first step showed the presence

TABLE I

YIELD OF BIS-CIBACRON BLUE AS A FUNCTION OF THE AMOUNT OF CONDENSING AGENT, CARBODIIMIDE

Yield is calculated on the basis of the extinction coefficient.

	Quantity (m*M*) of reagent			Bis-dye yield (m*M*)
	Cibacron Blue	Aminohexyl Cibacron Blue	Carbodiimide	
Preparation I	0.035	0.035	0	0
Preparation II	0.035	0.035	0.15	0.08
Preparation III	0.035	0.035	0.30	0.20
Preparation IV	0.035	0.035	0.45	0.17

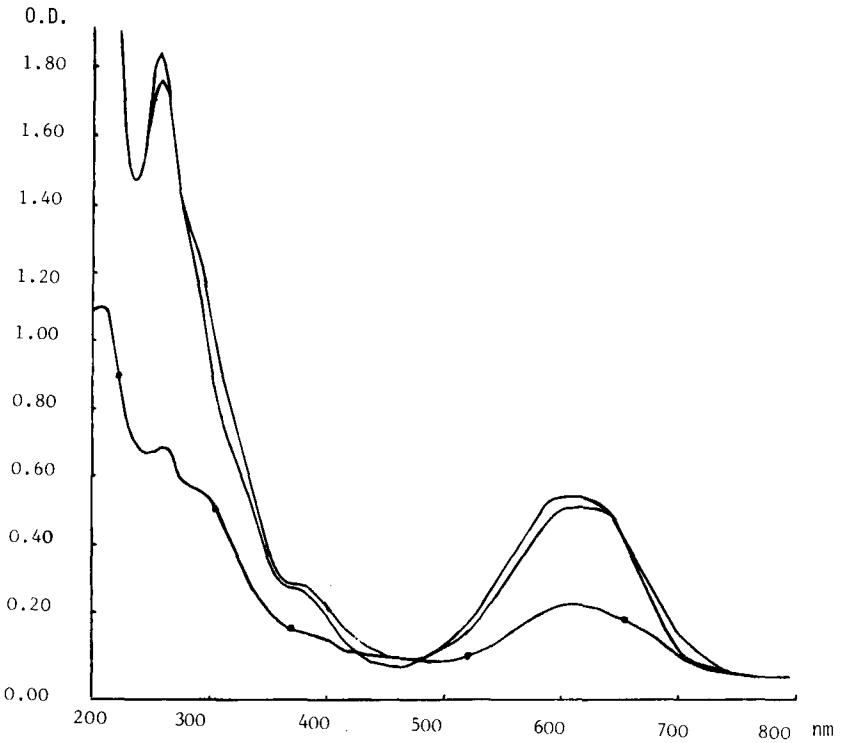

Fig. 2. UV—VIS spectra of the monomers and dimers of Cibacron Blue: (•——•) dimer; (——) monomer.

of the dimer in all cases, except in the absence of carbodiimide. The bis-derivative was recovered by column chromatography and by recycling the peaks. The UV—VIS spectra of the monomers and the dimers were compared and did not show any appreciable difference (Fig. 2).

Precipitation of the proteins

Table II shows the results obtained with LDH. It can be seen that 90% of the active enzyme was recovered in the precipitate, after specific dissociation of the complex, enzyme—bis-dye, using 0.1 m*M* NAD in the buffer.

TABLE II

AFFINITY PRECIPITATION OF LACTATE DEHYDROGENASE

See text for experimental details.

	Protein (mg)	Activity (I.U.)	Yield (%)
Starting solution	2	1105	100
Precipitate	1.8	677	90
Supernatant	0.2	16	10

TABLE III

COMPARISON OF THE PRECIPITATION OF LACTATE DEHYDROGENASE (LDH), BOVINE SERUM ALBUMIN (BSA) AND CHYMOSIN

	Yield with respect to initial solution (%)		
	LDH	BSA	Chymosin
Precipitate	90	50	20
Supernatant	10	50	80

Selectivity

Table III shows the results obtained with three different proteins: LDH, BSA and chymosin. As expected, LDH gives a 90% recovery, whereas only 50% BSA and less than 20% chymosin could be recovered. These results are in accordance with the results obtained in the case of dye—ligand affinity chromatography, where the dye monomer is coupled to the matrix [2, 5].

DISCUSSION

We have worked out a method for the synthesis of bis-Cibacron Blue via the formation of a sulphonamide compound; this could be represented as in Fig. 1. However, it is not very clear which of the sulphonic groups is involved in condensation with the amino group of the aminohexyl derivative. Our attempts to use NMR for this determination were not successful. Further studies are under way to determine the exact structure of the bis-dye. Nevertheless, it is very interesting to note that no precipitate was obtained with either the unreacted dye or the preparation containing the two dye moieties mixed in the absence of carbodiimide. Moreover, the precipitation of LDH was best achieved in the presence of pyruvate, the substrate of the enzyme. It is well known that cofactor NAD binds both to the enzyme and to the substrate. Hence, similar binding can be expected in the case of this biomimicking molecule, i.e. Cibacron Blue.

Contrary to the results observed by Pearson and Lowe [4], we did not observe any enhancement of the precipitation in the presence of multivalent metal ions such as CO^{+}. The metal, in fact, decreases the binding.

The results obtained with the different proteins tested, i.e. LDH, BSA and chymosin, could be directly correlated with those obtained from dye—ligand affinity chromatography, where the dye monomer is coupled to the matrix [2, 5]. In effect, little affinity toward chymosin was observed in our attempts to purify chymosin in a Cibacron-Blue-coupled matrix [6]. Thus, it is shown that the precipitation is affinity-oriented and not a non-specific aggregation of the protein.

Though these results are preliminary, they demonstrate the feasibility and the great potential of this new technique as a tool for the industrial purification of proteins based on affinity interactions.

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