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DYE LIGAND CHROMATOGRAPHY AND TWO-DIMENSIONAL ELECTROPHORESIS OF COMPLEX PROTEIN EXTRACTS FROM MOUSE TISSUE

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SUMMARY

A complex protein fraction of mouse brain was subjected to dye ligand chromatography with various dye ligands. The proteins that were bound by the dye–gel matrix and also the non-binding proteins were separated by high-resolution two-dimensional electrophoresis. The protein patterns obtained were compared. The results show that a large number of different protein species bind to dye ligands and do not occur in the eluate. Red A was the most efficient dye in isolating an individual protein class from a complex tissue extract. Moreover, we found that many of the binding proteins did not cross-react among different types of dye ligands. Orange A and Blue B were the most unrelated dyes among those compared. Our investigation shows that dye ligand chromatography can be used as a means (among others employed previously) of fractionating and classifying the enormous number of different protein species in a mammalian tissue when combined with high-resolution two-dimensional electrophoresis.

INTRODUCTION

In an attempt to analyse mouse proteins systematically, our strategy is to fractionate the proteins according to biological[1–3] or chemical[4] criteria and to separate the protein fractions by two-dimensional electrophoresis[5,6]. In this study, we tried to reveal, by using dye ligand chromatography of mouse brain proteins, classes of proteins characterized by their ability to bind to distinct dye ligands. This method uses sulphonated triazinyl dyes[7,8] as ligands. The most common dye used is Cibacron Blue 3GA (Ciba-Geigy), here referred to as Blue A (Amicon).

The mechanism of binding proteins to Blue A has been studied[9–12] and reviewed[13–16] by several investigators. Proteins that showed biospecific interactions with dyes are, in principle, enzymes that have negatively charged substrates, in particular phosphorylated substrates[15]. Enzymes using ATP, NAD and some other purine nucleotides have proved to be particularly strongly adsorbed by Blue A[17]. Stellwagen[9], studied the binding mechanism of lactate dehydrogenase and phosphoglycer-

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ate kinase and also the Blue A affinity of 43 other proteins and proposed the concept of nucleotide-fold specificity. However, other investigations have shown in a number of instances that proteins without a nucleotide-binding-fold bind to Blue A (data cited by Dean and Watson[13]). According to Miribel *et al.*[16], the fractionation of proteins by dye ligand chromatography results from different effects, such as ion exchange, diffusion–exclusion, pseudo-ligand affinity or hydrophobicity. This suggests that Blue A is a general ligand that interacts with several types of proteins. However, the binding specificity can be influenced by the conditions used to perform chromatography[11,18].

We performed dye ligand chromatography at a high pH, used short and wide columns and started with low ionic strength. Under these conditions, the total binding effect of the dye ligand may be decreased, while the relative portion of nucleotide-fold proteins that binds to Blue A may be increased.

EXPERIMENTAL

Preparation of solubilized cell proteins

Mice of the inbred strain DBA/2J (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were used as experimental animals. Investigations were carried out on 10–14-week-old females. Solubilized cell proteins (water-extractable proteins) were prepared from the brain. Ten brains were used for one preparation and two preparations were made. The method used for the preparation was the same as described earlier[2,4]. The frozen brains were thawed and homogenized (five up and down strokes, 250 rpm) in half a volume of deionized water. The homogenized tissue was centrifuged in a Beckman Ti-50 rotor for 40 min at 225 000 *g*. All of the fluid supernatant (including the thick and the clear layers) was removed and centrifuged again as above. The clear supernatant was considered as an extract that contains all protein species solubilized in the brain tissue. The extract was subjected to fractionation by heparin–Sepharose Cl-6B chromatography[4]. The proteins that did not bind to the heparin–Sepharose column were further subfractionated by dye ligand chromatography.

Dye ligand chromatography

Five dye ligand media were purchased from Amicon (Lexington, MA, U.S.A.): Blue A, Red A, Orange A, Green A and Blue B, coupled (by the triazine ring) to agarose as a supporting media. Prepacked columns (3.2 cm × 0.9 cm I.D.), available as a dye matrix screening kit, were used. The bed volume was 2 ml.

Before use, each matrix gel was regenerated with urea. A 12-ml volume of 8 *M* urea was added to each column, allowed to drain and stored overnight. The regenerated columns were equilibrated with 12 ml of a 50 m*M* Tris–HCl (pH 7.3) buffer (running buffer).

When Blue A, Red A or Green A was used, solutions containing 4.4 mg of protein were applied to the column; when Orange A or Blue B was used, 6.6 mg of protein were applied. The protein concentration was measured by the method of Lowry *et al.*[19] as modified by Peterson[20]. After an incubation time of 30 min, the column was washed with 15 ml of running buffer. Elution was achieved with a running buffer containing 1.5 *M* sodium chloride. The flow-rate of the solution was maintained at 5 ml/h. Fractions of 1.4 ml were collected. Two to three fractions of the

peaks absorbing at 280 nm were pooled. As a result, two pools were obtained, the binding and non-binding proteins of the sample. The pool containing the binding proteins was desalted and concentrated by Centricon (Amicon) ultrafiltration. The sodium chloride concentration was thereby reduced to about 1 m*M*, and the final protein content was in the range 4–12 mg/ml. The pool containing the non-binding proteins was concentrated in the same way. The final protein concentration was 4–16 mg/ml. For each dye type used, chromatography was performed twice.

The possibility that proteins are bound selectively to the ultrafiltration membrane (YM 10) of the Centricon tubes was investigated by comparing 2-DE patterns from unfiltered samples with those from diluted and reconcentrated aliquots of the same sample. The differences were negligible (0.3% of the spots showed qualitative changes and 3.8% of the spots showed quantitative changes).

Two-dimensional electrophoresis

The high-resolution two-dimensional electrophoresis technique developed by Klose[5] and modified by Klose and Feller[6] was used for final protein separation. This method combines isoelectric focusing in polyacrylamide gels containing urea and mercaptoethanol (first dimension) with electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS) (second dimension).

Protein samples were prepared from the pooled and concentrated fractions of the binding and non-binding proteins obtained by dye ligand chromatography. The protein fractions were mixed with urea, mercaptoethanol and ampholytes (pH 5–7) (Serva, Heidelberg, F.R.G.) to yield concentrations of 9 *M*, 5% (v/v) and 2% (m/v), respectively. The resulting mixture was stirred for 40 min at room temperature. Up to 60 μl of the sample containing about 150–220 μg of protein were applied to the gel. The samples prepared from the binding and non-binding protein fractions of the same dye column and separated by two-dimensional electrophoresis in parallel contained equal amounts of protein.

Isoelectric focusing (first dimension) was performed in a pH gradient generated by one part of Ampholine of pH 3.5–10 (LKB, Bromma, Sweden) and two parts of Servalyt of pH 5 7 (Serva). SDS gel electrophoresis (second dimension) was performed in 15% polyacrylamide slab gels. The size of the gels was 6.5 cm (running direction) × 7.4 cm × 0.31 cm. The proteins were stained with Serva Blue R250.

RESULTS

Evaluation of two-dimensional electrophoresis patterns

The protein patterns obtained by two-dimensional electrophoresis were compared with regard to the protein spot composition, *i.e.*, the positions of the spots of different patterns were compared. To facilitate this matching procedure, marker proteins were added to the protein sample. The following marker proteins were used: bovine serum albumin (Sigma), bovine β-lactoglobulin (Sigma, St. Louis, MO, U.S.A.), chicken conalbumin (Serva), bovine carbonic anhydrase (Serva) and horse myoglobin (Serva). A detailed description of the visual matching procedure was presented by Jungblut and Klose[4].

For each protein class investigated, two or three two-dimensional electrophoresis patterns were produced to ascertain the reproducibility of the spot number and

spot position. One of these patterns was used for evaluation and the repeat patterns were considered in cases of critical spots (*e.g.*, weak spots).

The two-dimensional electrophoresis protein patterns were evaluated with the aim of determining for each type of dye ligand chromatography performed the protein species unique to the binding proteins, unique to the non-binding proteins or common to both fractions. We then considered the binding proteins and compared the two-dimensional electrophoresis patterns of these proteins pair-wise. The two-dimensional electrophoresis patterns of each pair differed in the type of dye ligands from which they were deduced.

Binding proteins and non-binding proteins

The binding and non-binding protein fractions obtained by dye ligand chromatography using different dyes (Blue A, Red A, Orange A, Green A and Blue B) were separated by two-dimensional electrophoresis (Fig. 1). The patterns of the binding and non-binding proteins were compared. The protein spots common to both the binding and non-binding proteins were registered (Table I). The results show that Red A was the most efficient dye in isolating an individual protein class from the total protein sample. At best, the binding protein fraction contained only about 10% of proteins which also occurred in the non-binding fraction. In contrast, Blue B was the most inefficient dye for fractionating proteins. The portion of Blue B binding proteins that did not bind completely amounted to 34%.

When a complex protein mixture is fractionated by column chromatography into a binding and a non-binding fraction and the fractions are compared by two-dimensional electrophoresis, a general problem arises because these two fractions usually contain different amounts of proteins. The two protein samples used for two-dimensional electrophoresis can then be prepared from the binding and non-binding fractions in such a way that they contain (i) equal amounts of proteins or (ii) protein amounts differing in the same proportions as the protein amounts of the two fractions obtained from the column. We assume that the larger amount of proteins in the non-binding fraction compared with the binding fraction results from a larger number of protein species in that fraction rather than from a small number of protein species present in very high concentrations. If so, the excess of protein species in the non-binding fraction would result from protein species that do not bind completely (100%) to the column (protein species specific for the non-binding fraction). The number of protein species that do not bind completely to the column and, therefore, also occur in the non-binding fraction, is necessarily the same in the binding and non-binding fractions. However, common protein species may be distributed over these two fractions in different proportions, *e.g.*, 50% binding–50% non-binding; 90% binding–10% non-binding; 10% binding–90% non-binding. The consequence is that protein spots present in concentrations below the detection level in one two-dimensional electrophoresis pattern but above this level in the other would be wrongly classified as specific proteins. This error cannot be avoided or minimized, regardless of the quantitative proportions in which the proteins are applied to the two-dimensional electrophoresis. In contrast, in order to reveal the protein species, specific for the binding and non-binding fractions, in the same numerical proportions in the two-dimensional electrophoresis patterns as present in the unfractionated protein mixture, the protein content in the samples used for two-dimensional electrophoresis

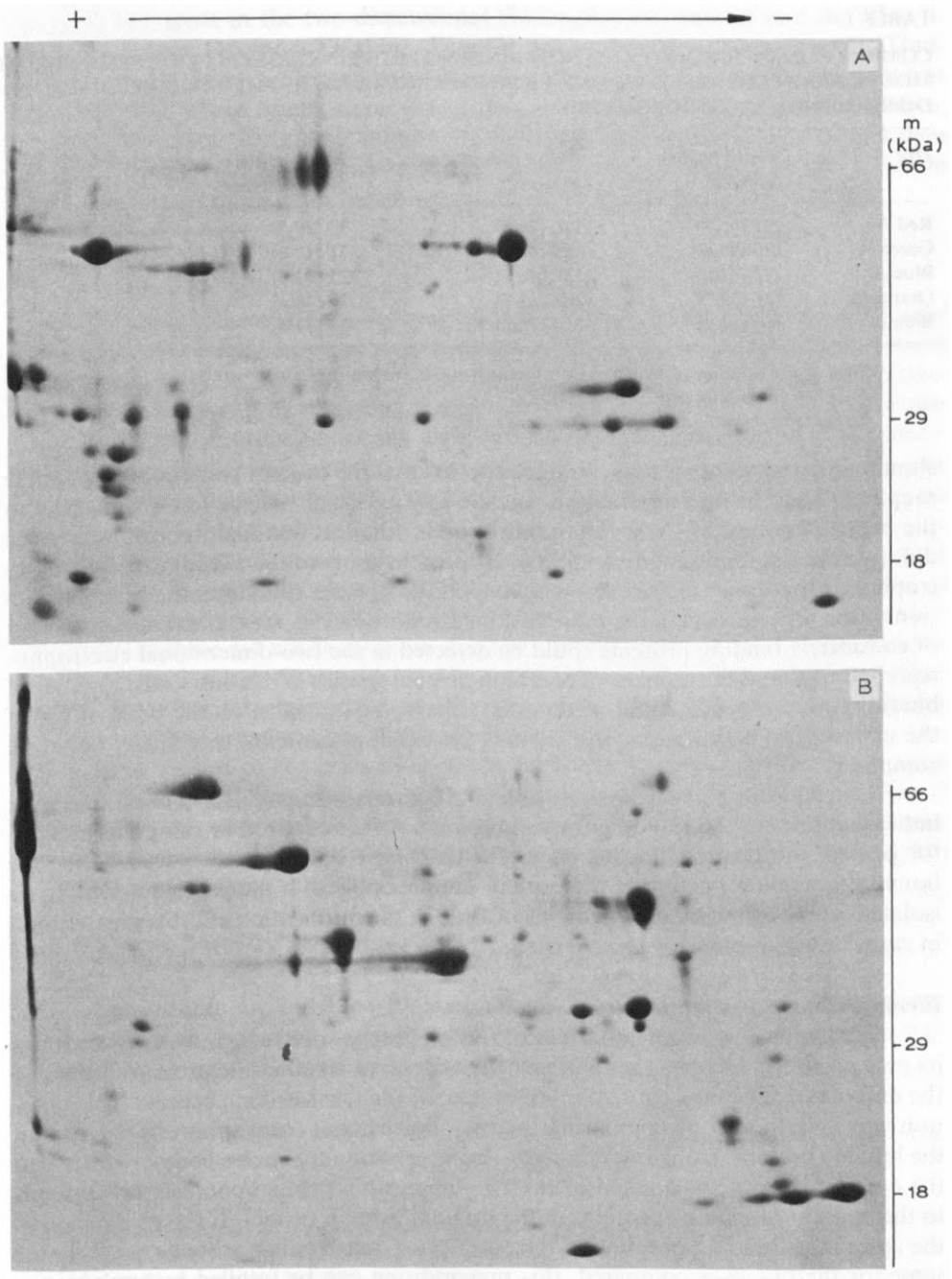

Fig. 1. Two-dimensional electrophoretic protein patterns from chromatographic fractions of mouse brain proteins. The solubilized brain proteins were subjected to heparin–Sepharose chromatography and the non-binding proteins were further fractionated by dye ligand chromatography using Red A. The protein fractions obtained were separated by two-dimensional electrophoresis. (A) Red A non-binding proteins; (B) Red A binding proteins. Small gels [6.5 cm (running direction) × 7.4 cm × 0.31 cm] were used for electrophoretic separation and Serva Blue R250 for protein staining. The patterns show considerable differences between the binding and non-binding proteins. m = Molecular weight; kDa = kilodaltons.

TABLE I

COMPARISON OF BINDING AND NON-BINDING PROTEINS (POLYPEPTIDE SPOTS) FROM MOUSE BRAIN OBTAINED BY DYE LIGAND CHROMATOGRAPHY FOLLOWED BY TWO-DIMENSIONAL ELECTROPHORESIS

Dye	*No. of binding proteins*[a]	*No. of non-binding proteins*[a]	*No. of proteins common to both fractions*[b]
Red A	287 (66.3)[c]	116 (26.8)[c]	30 (10.5)[c]
Green A	176 (47.6)	163 (44.1)	31 (17.6)
Blue A	173 (51.6)	137 (40.9)	25 (14.5)
Orange A	141 (29.7)	308 (64.8)	26 (18.4)
Blue B	156 (21.7)	510 (70.9)	53 (34.0)

[a] Sum of the number of binding, non-binding and common proteins = 100%.
[b] Number of binding proteins = 100%.
[c] Percentages in parentheses.

should differ correspondingly. In our experiments, the protein content in the sample prepared from the binding fraction was too low when the proportion with respect to the protein content of the non-binding protein fraction was maintained, and hence did not reveal a representative number of protein spots in the two-dimensional electrophoresis patterns. Therefore, we increased the protein content in the binding fraction to the protein level in the non-binding fraction. In this way, a maximum number of completely binding proteins could be detected in the two-dimensional electrophoresis patterns, and the number of common protein species in relation to the number of binding protein species could be used as a characteristic value on the basis of which the capacity of the different dye ligands for binding proteins specifically could be compared.

Whereas our aim was to use dye ligand chromatography as a method to obtain individual protein classes, other investigators may be interested in using this method for protein purification. In this respect Blue B may be preferred, because this dye bound the smallest portion of the protein sample applied. If the protein species to be isolated is among the binding proteins of Blue B, the purification effect is greater than in cases in which other dyes were used.

Binding proteins from different dye ligands

Let us consider whether each of the different types of dye ligand tested separates its own protein class from the total protein sample, or whether the proteins bound by the different dye ligands consist, more or less, of the same protein species. This question was investigated by comparing the two-dimensional electrophoresis patterns of the binding proteins from different dyes. A precondition for such a comparison is that the amount of proteins applied to the two-dimensional electrophoresis corresponds to the amount of binding proteins in the original protein extract. If the proportion of the amount of binding proteins to the amount of non-binding proteins is nearly the same for the two dyes compared, this precondition can be fulfilled by applying for each of the two dyes equal amounts of proteins to the two-dimensional electrophoresis. Under these conditions we were able to compare the following dyes: Red A–Green A, Red A–Blue A, Green A–Blue A and Orange A–Blue B. Among these, the most unrelated dyes were Orange A and Blue B. The Orange A-binding proteins

revealed 141 spots in the two-dimensional electrophoresis pattern and the Blue B-binding proteins 156 spots. Of these, 57 spots were common to both patterns. Thus, more than 60% of the protein spots of these two-dimensional electrophoresis patterns were different. These results show that different dyes bind different protein species, and that this is true for a large number of proteins. This suggests that different dyes act by different binding mechanisms and, therefore, may be used as one criterion among others for classifying the enormous number of unknown cell proteins.

CONCLUSION

Dye ligand chromatography and column chromatography in general are used to separate and purify single protein species or a few closely related protein species from a pool of other proteins[13,21]. The sample subjected to chromatography is usually already the result of preceding fractionation procedures. The investigation presented here demonstrates a basically different concept in the application of chromatography. Complex protein extracts were subjected to chromatographic fractionation and the aim was to isolate from the pool a particular protein class rather than a single protein species. Electrophoretic techniques were then used to characterize the separated protein fraction as an individual protein class. In this way, the investigation showed that dye ligands are able to bind a large number, possibly hundreds, of different protein species. Most of the binding proteins were not detected in the eluate and many of these did not show cross-reactions among different types of dye ligands. This suggests that proteins which bind to a certain type of dye ligand form a distinct class of proteins characterized by their binding mechanism(s). Studies on this mechanism would be of great interest. If this mechanism is a biospecific binding mechanism, the protein species of the class in question may also be characterized by common functional features. However, such studies may also reveal that a certain class of binding proteins consists of several subclasses characterized by biospecific and non-biospecific bindings. The use of other criteria to characterize protein classes[1–4] may help to elucidate these subclasses.

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