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Note

Affinity electrophoresis in an isotachophoretic discontinuous buffer system

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Affinity electrophoresis[1,2] is based on a combination of electrophoretic separation and specific interaction with an immobilized ligand incorporated into the gel support medium for electrophoresis. Various modifications of affinity electrophoresis can be used for detection and identification of ligand-binding proteins, verifying the homogeneity of the binding proteins and estimation of the strength of protein–ligand interactions. It is usually necessary to employ conditions under which the mobility of the protein is sufficiently high for the difference in mobilities observed on the control (non-interacting) gel and on the affinity gel to be great enough (at least 50%). Also, the presence of various protein impurities which do not interact with the ligand may obscure the results of an affinity electrophoresis, especially, when the specific ligand-binding protein is only a minor component of the sample and when some of the "impurities" have low mobilities. In the present communication we demonstrate the applicability of affinity isotachophoresis (AITP) which in some cases may be useful in avoiding these complications.

Isotachophoresis of proteins in gel media[3] is based on the choice of leading and terminating ions, so that the mobility of all charged molecules in the sample is higher than that of the terminating ion and lower than that of the leading ion. In such a system the proteins present in the sample will migrate within a stack between the boundaries of the zones of leading and terminating ions and will be separated into individual tandemly arranged zones according to their mobilities within the stack. If no "spacer" substances are added [such as ampholyte isoelectric focusing (IEF) carriers] which may aid in the separation of the individual protein components, the experimentally observable result after protein fixation and staining is that the sample migrates as a single sharp zone usually with little or no resolution of the individual components.

We expected that if isotachophoresis were to be performed in a gel containing

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immobilized ligand capable of complex formation with a component ot the sample, this component would, as a result of the interaction, leave the stack and emerge near the top of the gel. The following results verify this expectation.

MATERIALS AND METHODS

Materials

Agarose L was obtained from LKB (Bromma, Sweden) and chemicals for polyacrylamide gel preparation from Serva (Heidelberg, G.F.R.). Soluble O-glycosyl polyacrylamide copolymers were prepared by copolymerization of acrylamide and allyl glycosides of the respective sugars[4]; the carbohydrate contents of the α-D-mannosyl- and α-D-galactosyl copolymers were 14.1 and 6.7%, respectively. Crude pea seed lectin (PSA) was the protein fraction precipitated from pea seed meal extract by $(NH_4)_2SO_4$ (350 g/l); crude soybean lectin (SBA) was the protein fraction precipitated from soy bean extract by $(NH_4)_2SO_4$ (650 g/l). Heterogeneity of the samples was revealed by IEF performed in 5% polyacrylamide gel[5] containing 2% Ampholine 3.5–10 (LKB).

Isotachophoresis

Separating gels (60 × 2.5 mm; either 2% agarose L or 5% polyacrylamide gel) contained 0.125 *M* Tris–HCl pH 6.8. Affinity gels also contained corresponding O-glycosyl polyacrylamide copolymers (up to 5 mg/ml) and in some cases also free sugars. The electrode buffer contained 25 m*M* Tris and 192 m*M* glycine. Samples (20 μg of the protein) were loaded on the top of the gels in 20 μl of 25 m*M* Tris–HCl pH 6.8, containing glycerol (0.1 ml per ml) and bromophenol blue (10 mg/l). Isotachophoresis was performed at 60 V until bromophenol blue reached a distance of approximately 50 mm from the top of the gel. Proteins were stained by Coomassie Blue R 250.

RESULTS

The results of PSA and SBA are shown in Figs. 1 and 2. Clearly, PSA interacts specifically with α-D-mannosyl residues immobilized in the agarose gel whereas inactive impurities present in the sample remain in the stack migrating with the dye front. Similarly, SBA is retarded due to its interaction with α-D-galactosyl residues immobilized in the polyacrylamide gel. SBA specifically interacts also with the unmodified agarose gel matrix and this interaction can be abolished by free D-galactose (Fig. 1b). The degree of PSA or SBA retardation in affinity gels is dependent on the concentration of immobilized ligand (Fig. 2). The PSA isolectins are well resolved on the affinity gels containing lower concentrations of immobilized α-D-mannosyl residues.

DISCUSSION

In the present communication we have demonstrated the applicability of AITP to two lectins interacting with carbohydrates immobilized in affinity gels. This variant of affinity electrophoresis may possess certain advantages for qualitative detection of

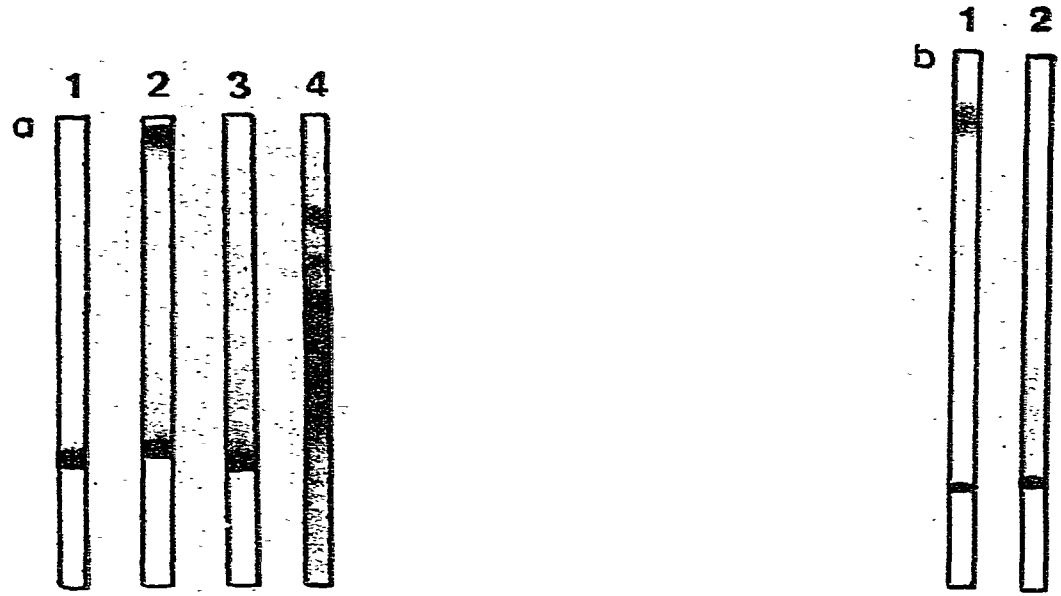

Fig. 1. Affinity isotachophoresis of PSA and SBA. a, Crude PSA on 5% polyacrylamide gels. 1 = Control gel; 2 = affinity gel containing 1% α-D-mannosyl polyacrylamide copolymer; 3 = the same as gel 2 but also containing 2% D-mannose; 4 = isoelectric focusing of crude PSA (for demonstration of the degree of heterogeneity of the sample). Note: Similar results to those obtained in gels 1–3 were also found with 2% agarose L instead of polyacrylamide gels (not illustrated). b, Crude SBA on 2% agarose gel. 1 = Pure 2% agarose L gel; 2 = as in 1 but the gel also contained 2% D-galactose.

ligand-binding proteins. Thus, all the inactive ballast proteins migrate rapidly in a single zone with the dye front and the specific binding protein is "extracted" from this single band on the affinity gel so that it is localized near the top of the gel. There is no interference with zones of ballast proteins in this region and the specific protein may be clearly identified, presumably even if present only in relatively low amount in the crude mixture. The interaction with immobilized ligand is clearly observable because of the marked difference in the rate of electrophoretic migration on control and affinity gels, respectively. Isotachophoresis can be used to "force" a protein to migrate relatively long distances in a control gel; this should be favourable for quantitative estimation of the strength of interaction of the protein with the immobilized ligand, but the fact that the protein migrates under different ionic conditions in the control gel (*i.e.*, within the stack) and in affinity gels (in terminating buffer) will probably affect the accuracy of such measurements. Thus, AITP is suggested for qualitative analytical purposes, similarly to affinity isoelectric focusing[6].

Although the technique clearly works in the above examples, it remains to be seen what are its limitations. For example, we were not able to demonstrate an interaction of defatted bovine serum albumin with immobilized Cibacron Blue F3GA (*i.e.*, affinity gels containing Blue Dextran T 2000) in the ITP buffer system used in this study (not illustrated), whereas specific interaction of the same protein with this ligand could be clearly demonstrated by affinity electrophoresis[7,8]. It seems likely that the removal of the protein from the stack and its retardation in affinity gel as a result of its interaction with immobilized ligand will depend on the actual balance between the counteracting effects of electrophoretic mobility of the protein, stacking properties of the buffer system and the strength of the protein–ligand interaction.

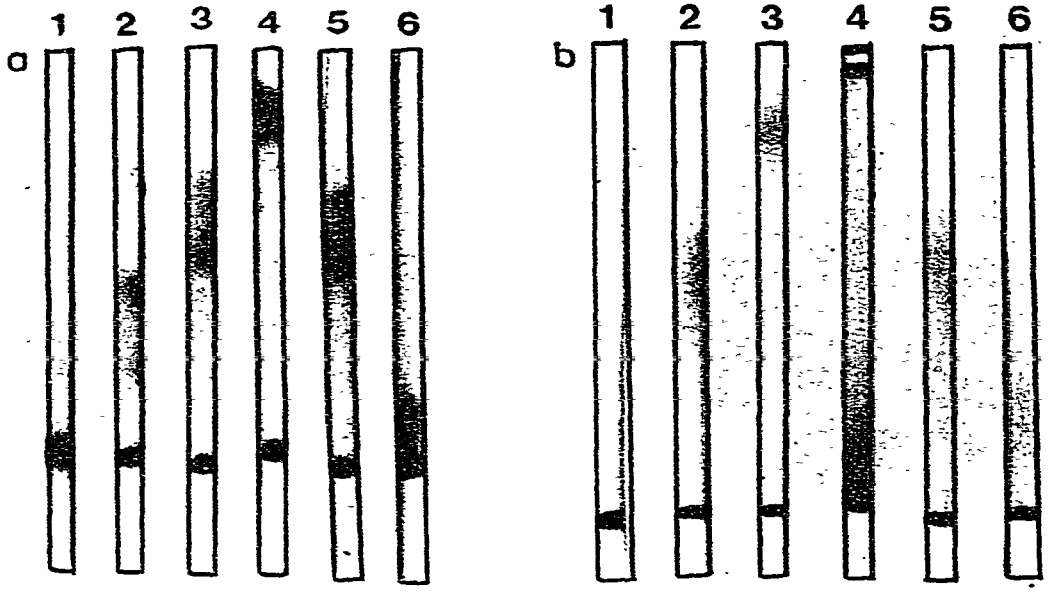

Fig. 2. Dependence of specific retardation of PSA (a) and SBA (b) on the concentration of immobilized and free sugars in 5% polyacrylamide gel during AITP. a: 1–4 = Gels containing 0, 0.1, 0.2 and 0.5% α-D-mannosyl polyacrylamide copolymer, respectively; 5, 6 = affinity gels containing 0.5% α-D-mannosyl polyacrylamide copolymer and 0.5 or 1% free D-mannose, respectively. b: 1–4 = affinity gels containing 0, 0.2, 0.5 and 1% α-D-galactosyl polyacrylamide copolymer, respectively; 5, 6 = affinity gels containing 1% α-D-galactosyl polyacrylamide copolymer and 0.5% or 1% free D-galactose, respectively.

We are aware that there may be formal objections to the designation of the technique as "affinity isotachophoresis" because the zone of the protein interacting with the immobilized ligand leaves the stack and migrates under the conditions of zone electrophoresis, while only the non-interacting proteins migrate isotachophoretically. However, we feel that this designation, although possibly inaccurate, correctly reflects the combination of the principles of isotachophoresis and biospecific affinity interaction which is the rationale of this method.

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