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AFFINOPHORESIS OF PEA LECTIN AND FAVA BEAN LECTIN WITH AN ANIONIC AFFINOPHORE, BEARING *p*-AMINOPHENYL-α-D-MANNOSIDE AS AN AFFINITY LIGAND

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SUMMARY

Affinophoresis is an electrophoretic separation technique for biological polymers with the aid of an affinophore, which is a macromolecular polyelectrolyte bearing affinity ligands. The affinophore migrates rapidly in an electric field, and consequently the electrophoretic mobility of molecules having an affinity for the ligand is specifically changed. An anionic affinophore-bearing mannosyl residue was synthesized for the affinophoresis of lectins. *p*-Aminophenyl-α-D-mannopyranoside and aminomethanesulphonic acid were coupled to about one-tenth and one-fifth, respectively, of the carboxyl groups of succinyl-poly-L-lysine with an average degree of polymerization of 120 by the use of a water-soluble carbodiimide. Extracts of seeds of pea (*Pisum sativum*) or fava bean (*Vicia fava*) were subjected to two-dimensional agarose gel electrophoresis, in which the first dimension was ordinary agarose gel electrophoresis and the second dimension was affinophoresis with the affinophore. The separated proteins were stained with Coomassie Blue R250. The lectins in both seed extracts were separated from a diagonal line formed by other proteins in the extracts. About 10 ng of the separated pea lectin was detected on a nitrocellulose blot by immunostaining with a horseradish peroxidase-conjugated second antibody.

INTRODUCTION

Affinophoresis is a separation method for biological polymers, based on their specific binding affinity[1–5]. The method employs a carrier molecule, an affinophore, which is specific for the molecules to be separated. The affinophore is a polyionic polymer bearing an affinity ligand(s). As the affinophore is highly positively or negatively charged, it migrates rapidly in an electric field. Molecules that have an affinity for the ligand form complexes with the affinophore and, consequently, their apparent electrophoretic mobilities are changed. Separation of molecules can be accomplished on the basis of this change in electrophoretic mobility.

The method is unique in that it utilizes a moving affinity ligand, not an immobilized ligand as used in most affinity techniques. Accordingly, the method permits the separation of electrically neutral substances by electrophoresis. Further, its appeal

as a general separation technique with possibly wide applications is due to the structural separation of the two functions of the affinophore, *i.e.*, the part which exerts affinity and the part which causes migration in the electric field. Therefore, the method should be readily applicable to many molecules with different binding specificities simply by changing the affinity ligand on an affinophore matrix.

Affinophores have been prepared by using several kinds of polyelectrolytes as matrices, *e.g.*, diethylaminoethyldextran for a cationic affinophore[1] and polyacrylyl-β-alanyl-β-alanine[3] and succinyl-poly-L-lysine[4] for anionic affinophores. Recently, the combination of affinophoresis with two-dimensional agarose gel electrophoresis has proved to be very effective for the isolation of specific proteins from complex mixtures[5]. The procedure can be completed within 2 h without costly special equipment.

We have demonstrated the utility of affinophoresis in the separation of proteases[1–5]. In this work, we applied affinophoresis to the separation of lectins. Lectins are proteins or glycoproteins having carbohydrate-binding ability except for sugar-specific enzymes and anti-carbohydrate antibodies. They have been found in a wide range of species from bacteria to mammals and most abundantly in legume seeds, but their role in living organisms is still unclear. *p*-Aminophenylglycosides have been used as potent affinity ligands in the affinity chromatography of lectins[6]. An anionic affinophore for mannose-binding lectins was prepared, using *p*-aminophenyl-α-D-mannoside as an affinity ligand and succinyl-poly-L-lysine as an affinophore matrix. Lectins were isolated from extracts of seeds of pea and fava bean by affinophoresis with this affinophore.

EXPERIMENTAL

The sources of materials used in this work were as follows: poly-L-lysine hydrobromide (average degree of polymerization 120) and *p*-aminophenyl-α-D-mannopyranoside from Sigma (St. Louis, MO, U.S.A.); purified mannose-binding lectins from pea (*Pisum sativum*) and fava bean (*Vicia fava*) from Hohnen Oil (Tokyo, Japan); rabbit immunoglobulin G (IgG) fractions against pea lectin (PSA) from E. Y. Labs. (San Mateo, CA, U.S.A.); horseradish peroxidase-conjugated goat IgG (anti-rabbit IgG) from Miles Labs. (Elkhart, IN, U.S.A.): and SeaKem agarose (ME) and Gel Bond film from FMC, Marine Colloids Division (Rockland, ME, U.S.A.). Seeds of pea and fava bean were obtained from a local seed shop.

Preparation of extracts of seeds

Meal of the seeds of pea and fava bean was prepared by the use of an electric sample mill with rotating blades. Each meal sample (0.5 g) was defatted by extraction with acetone (2 ml, 3 × 10 min) and dried *in vacuo*. It was then stirred with 2.5 ml of 0.15 *M* sodium chloride solution containing 0.02% sodium azide for 3 h at room temperature. The mixture was centrifuged for 10 min at 10000 *g* and 4°C. The supernatant was used as the extract.

Preparation of affinophore

Succinyl-poly-L-lysine was prepared by the reaction with succinic anhydride, as described previously[4], and dialysed against 0.1 *M* sodium chloride solution. To

the solution of succinyl-poly-L-lysine (5 ml) containing 334 μmol of lysine residue, *p*-aminophenyl-α-D-mannopyranoside · $1.5H_2O$ (10 mg, 34 μmol) was added and the pH was adjusted to 4.75 with 1 *M* hydrochloric acid. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (32 mg, 170 μmol) was added at once and the mixture was stirred for 10 min. Almost all the *p*-aminophenyl-α-D-mannoside was bound to the polymer [checked by UV absorption measurement after thin-layer chromatography (TLC) on silica gel; R_F 0.63; ethanol–water (9:1, v/v)]. After a further 10 min, aminomethanesulphonic acid (7.4 mg, 67 μmol) and the carbodiimide (32 mg, 170 μmol) were added and the pH was maintained for 20 min between 4.5 and 5.0 with 1 *M* sodium hydroxide solution. The pH was then adjusted to 7 with 1 *M* sodium hydroxide solution, and the solution was dialysed against 0.1 *M* sodium chloride solution (1 × 1 l) and water (2 × 1 l). The purified preparation (9.0 ml) was used as a stock solution of affinophore, containing 31 m*M* of lysine residue and 2.9 m*M* of mannoside. The lysine content was determined by amino acid analysis[4] and the content of mannoside was determined by the phenol–sulphuric acid method, using *p*-aminophenyl-α-D-mannoside as a standard.

The electrophoretic mobility of the affinophore was determined by zone electrophoresis in 1% agarose gel (1 mm thick) containing 0.1 *M* tris(hydroxymethyl)-aminomethane (Tris)–acetic acid buffer (pH 7.9). The stock solution of the affinophore was concentrated 5-fold by evaporation, and 2 μl of the solution were subjected to electrophoresis for 30 min at 25 V/cm under cooling with ice-cold water. After electrophoresis, a section of the agarose gel, containing the affinophore, was transferred on to a silica gel plate containing fluorescent indicator for TLC. The migration of the affinophore was observed under UV light. The affinophore migrated to a position 5.5–6.5 cm anodal from the origin, while bromophenol blue, which was subjected to electrophoresis simultaneously, migrated 5.0 cm.

Affinophoresis

Two-dimensional affinophoresis was carried out on a 1% agarose gel plate (1 mm thick) containing 0.1 *M* Tris–acetic acid buffer (pH 7.9) as described previously[5]. Briefly, a square gel plate (8 × 8 cm) was formed on a Gel Bond support film (FMC) (8 × 12.5 cm), leaving an open area of 8 × 4.5 cm, by spreading 6.5 ml of 1% agarose solution in the buffer. The first electrophoresis was carried out for 30 min at 25 V/cm (40–50 mA per plate) under cooling with ice-cold water in a direction parallel to the shorter edges of the support film. Sample (2 μl) was applied in a hole (1.5 mm diameter) made at a point 2.5 cm from the edge of the gel facing the open area of the film and 3.5 cm from the edge facing the cathode. The voltage gradient was monitored at the surface of the gel. After the first electrophoresis, the part of the gel (2 cm width) that faced the open area of the film was cut away. On the enlarged open area of the film was spread 5 ml of 1% agarose solution, containing 0.1 ml of the affinophore stock solution in the buffer. Immediately after gel formation, a second electrophoresis was carried out under the same electrophoretic conditions as the first in a direction perpendicular to that of the first, with the side of the gel containing the affinophore facing the cathode. Methyl-α-D-mannoside (0.1 *M*) was included only in the first gel, when it was used. Proteins were stained with Coomassie Brilliant Blue R250, as before[5].

Immunostaining

After electrophoresis, the gel was covered with a sheet of nitrocellulose membrane (Millipore HAWP) and the membrane was discarded after thorough wetting. This procedure removed unknown material on the gel surface which causes spurious staining. Another nitrocellulose sheet was placed on the gel and covered with a sheet of wet filter paper and five sheets of dry blotting paper. Pressure was applied by means of a glass plate and a weight of 1 kg. The blotting paper was changed twice at 5-min intervals. The nitrocellulose sheet was peeled off and dried in a glassware dryer (50°C) for 15 min.

The following process was performed at room temperature unless indicated otherwise and with gentle shaking on a shaker. The dried membrane was immersed in 25 ml of a blocking solution, which contained 1% (w/v) of food skim milk powder and 1% (w/v) of Tween 20 in TBS [10 m*M* Tris–HCl containing 0.9% sodium chloride (pH 7.4)] for 15 min with changes of the solution at 5-min intervals. The membrane was treated overnight at 4°C with rabbit anti-lectin antibody at a dilution of 1:1000 in 15 ml of the blocking solution. It was washed three times with 20 ml of the blocking solution (5 min each) and treated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody at a dilution of 1:1000 in 15 ml of the blocking solution. After washing with the blocking solution (3 × 25 ml, 5 min each) and TBS (2 × 25 ml, 5 min each), lectin was revealed by the enzyme reaction performed for about 10 min in a mixture of 3 ml of 0.3% (w/v) 4-chloro-1-naphthol in methanol, 15 ml of TBS and 0.18 ml of 1% (w/v) hydrogen peroxide[7]. The membrane was finally washed with water.

The detection limit of immunostaining was examined after one-dimensional affinophoresis of a 2-fold dilution series of purified pea lectin and was found to be 10 ng.

RESULTS AND DISCUSSION

Affinophore for lectins

An affinophore for lectin was readily prepared by using poly-L-lysine as a matrix of the affinophore. This commercially available polymer is very useful for the preparation of affinophores[4]. The nucleophilic amino groups of the polymer enables various derivatizations to be carried out for the synthesis of affinophores. The concentration of the lysine residue can be determined by amino acid analysis. Its ionic character can be readily changed from cationic to anionic by complete succinylation.

In principle, affinophoresis can be carried out with either cationic or anionic affinophores. However, in practice, affinophoresis with a cationic affinophore has some disadvantages. First, cationic affinophores bind to the agarose gel used as an insoluble support for electrophoresis[1,2]. The binding presumably arises from ionic interaction with negative charges on the agarose. Second, cationic affinophores are deeply stained with the anionic dyes ordinarily used for protein staining, such as Coomassie Blue R250 or Amido Black 10B[1,2]. Therefore, the succinylated form of the polymer was used as the matrix of the affinophore for lectins.

For the affinity ligand, *p*-aminophenyl-α-D-mannopyranoside was used. *p*-Aminophenylglycosides have been used as potent affinity ligands in affinity chromatography of lectins[6] and can be readily coupled with succinyl-poly-L-lysine by the

use of water-soluble carbodiimide. The affinophore was also faintly stained with Coomassie Blue R250 dye, and this resulted in a dark background when it was used at high concentrations. The introduction of sulphonic acid groups makes the affinophore unstainable[3,4]. To introduce sulphonic acid groups, one-fifth of the carboxyl groups of the matrix were coupled with aminomethanesulphonic acid.

Affinophoresis of pea extract

Pea seed contains a lectin specific for D-mannose or D-glucose. The lectin has a molecular weight of 55000 and has two sugar-binding sites[8-10].

The extract of pea seeds was subjected to two-dimensional affinophoresis with the anionic affinophore-bearing mannoside, and the separated proteins were stained with Coomassie Blue R250 (Fig. 1A). The affinophore was used at a concentration of one-fiftieth of the stock solution, *i.e.*, 0.058 m*M* mannoside and 0.62 m*M* lysine residue. In this two-dimensional affinophoresis, proteins that have no affinity for the affinophore migrate to locations along a diagonal line passing through the origin, whereas proteins that have affinity are carried away from the line by the affinophore. A single spot was separated from the diagonal line. A streak running from the origin to the anode in the second dimension would be an artifact. A similar streak was also observed in the absence of the affinophore. Immunostaining of the blotted nitrocellulose sheet confirmed that the separated spot stained with Coomassie Blue R250 was pea lectin (Fig. 1B). In the presence of 0.1 *M* methyl-α-D-mannoside, which would bind competitively to the lectin, the spot was not separated from the diagonal line (Fig. 1C and D). Affinophoresis of purified pea lectin was also carried out under the same conditions. The lectin migrated to a position identical with that of the spot separated from the seed extract (Fig. 2). The results show that affinophoresis separated pea lectin from the extract and that the interaction between the affinophore and the lectin is based on specific affinity.

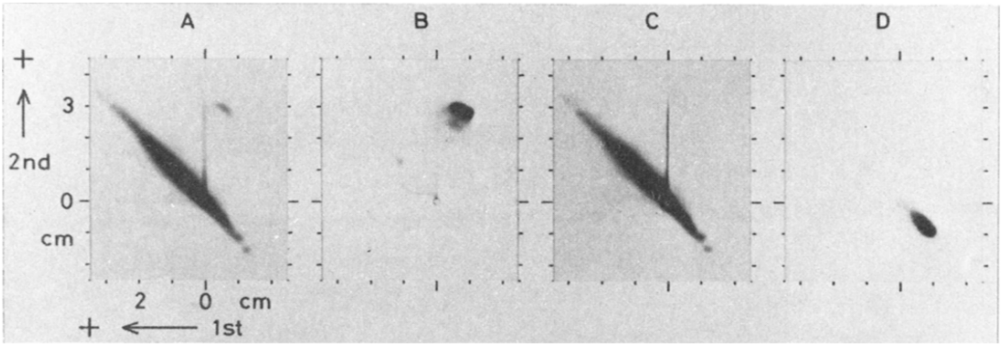

Fig. 1. Isolation of pea lectin from a seed extract. The extract of pea seed was applied at the positions marked O, and two-dimensional affinophoresis was carried out using the mannoside-bearing affinophore in the absence (A and B) or presence (C and D) of 0.1 *M* methyl-α-D-mannoside. A and C, 2 μl of the extract were applied and stained with Coomassie Blue R250; B and D, 2 μl of the 10-fold diluted extract were applied and immunostaining was carried out on the blotted nitrocellulose with rabbit anti-pea lectin antibody. Details of the two-dimensional affinophoresis and immunostaining are described in the text.

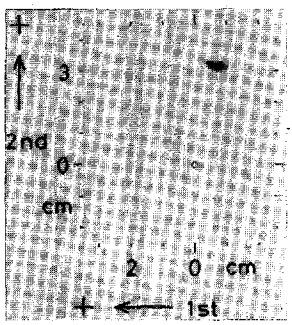

Fig. 2. Two-dimensional affinophoresis of purified pea lectin. Purified pea lectin (4 μg in 2 μl of 0.1 *M* Tris–acetic acid buffer, pH 7.9) was applied at the position marked ○, and two-dimensional affinophoresis was carried out under the same conditions as in Fig. 1A.

Affinophoresis of fava bean extract

Mannose-binding lectin has also been found in fava bean seed. The lectin has two sugar-binding sites, and its molecular weight is 50000[11,12]. Fava bean extract was subjected to two-dimensional affinophoresis under the same conditions as in the experiment in Fig. 1. A protein spot was separated from the diagonal line, and the effect of the affinophore was suppressed by the addition of 0.1 *M* methyl-α-D-mannoside in the gel (Fig. 3A and B). Purified fava bean lectin migrated to a position identical with that of the spot separated from the seed extract under the same conditions of the affinophoresis. Hence fava bean lectin was also separated from the seed extract by two-dimensional affinophoresis.

The greater mobility of fava bean lectin than that of pea lectin in the second dimension, *i.e.*, affinophoresis, should reflect the greater affinity of fava bean lectin than that of pea lectin to the mannoside-bearing affinophore. This is consistent with the reported binding constants of these lectins for D-mannose: 900 M^{-1} for fava bean lectin[12] and 140 M^{-1} for pea lectin[10].

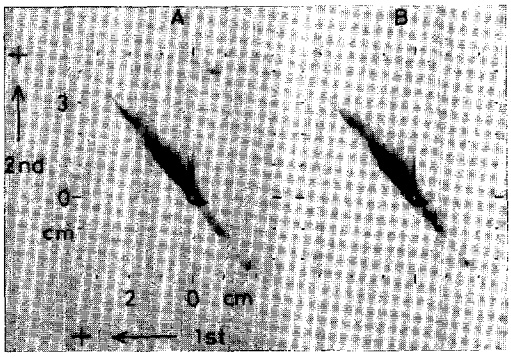

Fig. 3. Isolation of fava bean lectin from the extract of seeds. The extract (2 μl) of fava bean seeds was applied at the position marked ○, and two-dimensional affinophoresis was carried out by using the mannoside-bearing affinophore in the absence (A) or presence (B) of 0.1 *M* methyl-α-D-mannoside. Proteins were stained with Coomassie Blue R250.

Affinophoresis of jack bean extract

Jack bean contains a very extensively investigated mannose-binding lectin, concanavalin A. However, application of two-dimensional affinophoresis to jack bean extract did not afford clear results, owing to the formation of a precipitate of concanavalin A in the course of the affinophoresis. Experimental conditions suitable for separating concanavalin A are under investigation.

CONCLUSIONS

A highly specific separation of lectins from crude extracts of legume seeds was achieved by two-dimensional affinophoresis. Affinophoresis has been successfully applied to proteases, trypsins from several sources[1–3,5] and native and chemically modified chymotrypsin[4]. Recently, we separated a hapten-specific antibody from rabbit serum by using an affinophore bearing the hapten. Hence the method has general applicability to various types of specificity. Affinophoresis should be able to replace affinity chromatography in some fields of research and clinical diagnosis, providing more information with greater simplicity of operation in a shorter time.

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NOTE ADDED IN PROOF

It was found that the difficulty with jack bean extract was due to the outstanding abundance of the lectin in the bean. Concanavalin A was separated from the seed extract by applying affinophoresis to a 10-fold diluted extract.

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