Polyacrylamide Gel Affinity Electrophoresis for Separation of Enzyme Isoforms

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Abstract

Affinity electrophoresis of differently glycosylated isoforms of enzymes using lectin as affinity ligand has been reported on support media such as cellulose acetate membrane (CAM) or agarose gel. We report a method for affinity electrophoresis in polyacrylamide gel (PAG) using wheat germ agglutinin (WGA). WGA is added to acrylamide-Bis mixture and incubated for 10 minutes at room temperature. This causes WGA to react covalently with acrylamide and Bis. Polymerization is initiated with addition of N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulphate to give polyacrylamide gel with immobilized lectin. This gel has been found to effectively separate differently glycosylated isoforms of alkaline phosphatase (ALP). Concanavalin - A, similarly immobilized, did not give effective separation of ALP isoforms. The immobilization of lectin on polyacrylamide as support media requires less amount of lectin in comparison to CAM and agarose. Additional advantage of affinity electrophoresis on PAG is separation of biomolecules according to size.

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Key Words : Affinity electrophoresis; Alkaline phosphatase; Isoforms separation; Lectin; Polyacrylamide gel

Introduction

The non-genetic variations in post translational glycosylation are mainly responsible for different isoforms of ALP (E.C.3.1.3.1) in different tissues [1]. The separation of such differently glycosylated enzyme isoforms is difficult by conventional electrophoretic techniques as these have almost same charge and size. Currently available affinity electrophoresis techniques incorporate WGA in electrophoresis-buffer, which is then used for soaking the CAM [2] or casting the agarose gel [3]. In these techniques, both enzyme-lectin complex and lectin remain mobile and therefore, a large quantity of lectin is required.

Acrylamide binds covalently with haemoglobin and forms adducts with alpha and beta chains of haemoglobin in-vitro [4]. Unreacted acrylamide in gel is known to react with free-SH groups of proteins forming cysteinyl-S-propanamide [5]. Pretreatment of acrylamide gel with haemoglobin improves transfer of micro-quantities of proteins in Western blot [6]. In view of these known binding properties of acrylamide, this study was aimed at exploring whether lectins can be immobilized onto the polyacrylamide gel for affinity electrophoresis for separation of differently glycosylated enzyme isoforms. We were able to separate differently glycosylated isoforms of ALP by WGA immobilized onto PAG.

Material and Methods

Reagents : Acrylamide, Bis, wheat germ agglutinin, Concanavalin-A, glutaraldehyde and Tris were from Sigma Chemical Co. N,N,N',N'-tetramethylethylenediamine (TEMED) was from Eastman Chemicals. Other reagents

were of analytical grade from local sources.

Serum samples with high activity of ALP were collected from a pregnant woman, growing child, patients of hepatocellular, gallbladder and prostatic cancers. Different variations of affinity electrophoresis were experimented to find best conditions for effective separation of ALP isoenzymes (Table 1).

Table 1

Inclusion of lectin in polyacrylamide gel under various conditions and ALP isoform separation

Electrophoretic conditions	Results
No pretreatment of serum samples. Electrophoresis on 4-7% polyacrylamide gel.	Enzyme enters gel No separation of isoforms.
Pretreatment of serum samples (15 l) with Enzyme did not enter gel. 30 g of WGA or 75 g of Con-A and electrophoresis on 4-7% gel.	
No pretreatment of serum samples. Incorporation of Con-A in gel during polymerization.	Enzyme entered the gel No separation of isoforms.
No pretreatment of serum samples. Inclusion of WGA (62.5 g/mL) in gel during polymerization.	Effective separation of isoforms.
No pretreatment of serum samples. Inclusion of WGA (62.5 g/mL) in PAGE in presence of glutaraldehyde during polymerization.	Effective separation of isoforms.

Preparation of Polyacrylamide gel and electrophoresis :

5% PAG containing 62.5 g WGA/mL of gel was prepared by mixing 1mL of acrylamide-bis solution (38 g acrylamide and 2 g bis in 100 mL of water), 1mL of water and 2mL of solution containing 250 g of WGA/mL. The mixture was gently agitated and left for 10 minutes at room temperature for WGA to react with Bis/acrylamide. 2 mL of TEMED

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solution of (0.23mL of TEMED in 100mL of Tris-Boric acid Buffer pH 9.0) was added to the above mixture to initiate polymerization which was further catalysed by addition of 2mL of ammonium persulphate (4mg/mL). The solutions were mixed gently to avoid froth formation. In the early part of study, 1 mL of 0.02% glutaraldehyde, a bifunctional cross-linking agent, was also used to immobilize the lectin [7] in place of distilled water in the mixture described above for preparation of acrylamide gel. Con-A containing gel (625 g/mL of gel) was similarly prepared replacing WGA solution with Con-A solution (2500 g Con-A/mL). The gel slabs (10 cm x 10 cm x 1mm) were cast after degassing. PAG 4-7% without WGA were similarly prepared by varying concentration of acrylamide-bis solution and adding distilled water in place of WGA solution. Tris-boric acid buffer (0.375M) was prepared by dissolving 45.5 g of Tris in 900 mL of water and adjusting the pH to 9.0 or 9.5 with boric acid and volume to 1L with water.

The PAG electrophoresis was carried out in cold chamber at 4°C, initially at 30 volts for 10 minutes and thereafter at 90 volts for 60-70 minutes in constant voltage mode. ALP activity was detected by incubating the gel in dark at 37°C in solution containing alpha-naphthyl phosphate (20 mg) and Fast Blue BB (10mg) in 10 mL of buffer (Boric acid; 3.74g and MgCl₂, 6H₂O; 2.04 g adjusted to pH 9.7 with KOH and volume to 1L with water).

Results

The bone and liver isoforms of ALP did not separate on 4 to 7% PAG. We pretreated the serum samples to see whether the interaction with WGA or Con-A can effect separation of isoforms electrophoresed on PAG. The enzyme and proteins got agglutinated at higher concentration (WGA 30 g or Con-A 75 g on 15 L of serum) and did not enter the gel. However, when samples were pretreated at lower concentrations of WGA or Con-A, the enzyme isoforms did enter the gel in increasing concentrations but did not separate. Simply including WGA in acrylamide-bis mixture (125 g/mL) during polymerization effectively separated the bone and liver isoforms (Fig1). Similar results were obtained when glutaraldehyde was used to immobilize the lectin. This however was found to be redundant as lectin immobilization and isoform separation could be achieved without using glutaraldehyde. Separation of isoforms could not be achieved on Con-A containing gels nor did they reveal any different isozyme pattern (Table 1).

Discussion

Reaction of cysteine-SH group of proteins with double bond of acrylamide and bis forms cysteinyl-S-propionamide or cysteinyl-S-bis derivative, respectively, as suggested by Chiari [5]. The WGA when incorporated before polymerization similarly gets covalently bonded through -SH groups and on polymerization gets immobilized through pendant bis groups [6]. This on electrophoresis effects separation of differently glycosylated isoforms of ALP. The

Fig. 1 :

separation of bone and liver isoforms in PAG electrophoresis on incorporation of WGA was due to multiple interactions of glycosyl groups of bone isoform with WGA, which retarded the movement of bone isoform and led it to separate from nonglycosylated liver isoform.

The bone and liver isoforms did not separate in gels containing Con-A, nor did Con-A containing gels give any different ALP isoenzyme pattern. The reason for Con-A not being able to separate the ALP isoform could be that the pH or other experimental conditions were not ideally suited for differential interaction of Con-A with sugar chains [8].

Thus, affinity electrophoresis on PAG can be simply carried out by incorporating lectins during polymerization, where lectins through-SH groups react with double bond of bis at room temperature and get incorporated into the gel on polymerization leaving lectin molecule pendant to such bis molecules. The affinity electrophoresis on PAG could be effectively utilized for separation of proteins, which get differently glycosylated either normally or under disease conditions, such as alpha-feto protein [9] and gamma-glutamyl transpeptidase in hepatoma [10]. It is possible to incorporate other affinity proteins such as avidin or antigens/antibodies in polyacrylamide gel to separate proteins specifically interacting with them [11].

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ECG Quiz

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Clinical Information

36 year old female patient, a known case of rheumatic heart disease with mitral and aortic regurgitation (closed mitral valvotomy done for mitral stenosis 7 years ago), on digoxin, diuretics, oral potassium and erythromycin prophylaxis, was admitted with severe

dyspnea and palpitations at rest. Clinically patient had irregular heart rate above 150/min, blood pressure 80/60 mm Hg and pulmonary edema. Electrocardiogram before and after 250 mg intravenous phenytoin sodium is shown below. What is your diagnosis?

Fig. 1 :

Answer to the ECG quiz - pg. 165

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