Identification of Rat Serum Alkaline Phosphatase Isoenzyme by means of Wheat Germ Agglutinin

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Abstract: Wheat germ agglutinin (WGA) precipitates bone type serum alkaline phosphatase (sALP) isoenzyme specifically. The precipitates are composed of the macromolecules of WGA and “bone type sALP” (WGA-ALP complex). In order to use bone type sALP as a marker in polyacrylamide gel electrophoresis (PAGE), a method to separate “bone type sALP” from the “WGA-ALP complex” was established by using N-acetyl-D-glucosamine (GlcNAc)-Sepharose 6B column chromatography. It was concluded that this method is useful for clinical examination in the rat.

Key words: rat, serum alkaline phosphatase (sALP), wheat germ agglutinin (WGA)

When using markers extracted from various tissues to identify the origin of sALP isoenzymes on polyacrylamide gels, much attention should be paid to the changes in glucosylated chains occurring at the time of tissue extraction and contaminating proteins. In addition, the solubilization of membrane-bound tissue ALP in the laboratory with n-butanol is not physiological and undoubtedly cleaves ALP from the cell membrane at a different site than in vivo [4]. If markers obtained from serum isoenzymes could be used to identify the tissue of origin of sALP isoenzymes by PAGE, the marker would be useful for clinical examination.

WGA precipitates “bone type sALP” specifically [1, 5]. The precipitates are composed of macromolecules of WGA and “bone type sALP” (WGA-ALP complex). They cannot pass into the polyacrylamide gel, so that the precipitates themselves cannot be used as a marker in PAGE. The purpose of this study was to establish a method to separate “bone type sALP” from the “WGA-ALP complex”.

Six-week-old male Crj:CD (SD) rats (Charles River Japan Inc., Yokohama, Japan) were used. They had free access to a chow diet (CE-2, Clea Inc., Tokyo, Japan) and tap water. Blood was taken from the orbital venous plexus under light anesthesia with ether. Serum was obtained by centrifugation at 1,500 × g and stored at −40°C until used.

WGA was purified in our laboratory from crude wheat germ (Sigma Chemical Co., MO) according to the method of Vretblad [7]. 150 µl of bovine serum albumin solution (50 mg/ml) containing 5 mg/ml WGA was added to the rat serum (150 µl). After incubation [5]...
at 37°C for 30 min, the mixture was centrifuged at 37°C for 10 min at 15,000 × g, and the precipitate was subjected to column chromatography.

A GlcNAc-Sepharose 6B column was prepared according to the method of Vretblad [7] by using epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden). The column (2.0 × 6.5 cm) was equilibrated with 0.05 M sodium acetate buffer (pH 4.5). The precipitate was dissolved in 400 µl of 0.05 M sodium acetate buffer (pH 4.5) and applied to the column. The column was then washed with the same buffer solution. The protein having ALP activity (ALP-protein) was released with a linear gradient of 0–0.1 M GlcNAc solution.

Samples of rat serum, the supernatant and the precipitate, obtained after WGA treatment, and the eluate obtained by GlcNAc-Sepharose 6B column chromatography were subjected to PAGE. Electrophoresis was performed with Ready Gels J (5%–10%, Bio-Rad Lab., Hercules, CA) under nonreducing conditions in a BIO-RAD Mini-Protean II electrophoresis Cell Apparatus (Bio-Rad Lab.) with 90 mM Tris/borate-sodium dodecyl sulfate (SDS, 0.1%) buffer (pH 9.0), in the buffer compartments. ALP activity was not inhibited by SDS up to at least 3% (data not shown). Equal volumes of samples (0.5–20 µl) were electrophoresed at 10 mA/gel for 180 min at 4°C in each experiment. After electrophoresis, ALP activity was demonstrated by incubating the gel for 60 min at 37°C with a chromogenic substrate, 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (NACALAI TESQUE, Kyoto, Japan) at a final concentration of 1.25 mM, in a 0.1 M of 2-amino-2-methyl-1,3-propanediol buffer (pH 10.2) and 1 mM of magnesium sulfate [8]. The stained gel was then soaked overnight in aqueous acetic acid (50 ml/l), washed with distilled water and dried.

Gels on which the molecular weight (M.W.) standards were electrophoresed were fixed for 60 min with 12.5% trichloroacetic acid. The fixed gel was then stained for 60 min in aqueous CBB R-250 methanol/acetic acid, and then decolorized in 7.5% acetic acid for 24 hr [6].

SDS-PAGE Protein Standards High Range (Bio-Rad Lab.) was used as a M.W. marker.

ALP activity was assayed at 37°C with disodium p-nitrophenyl phosphate (PNP, NACALAI TESQUE) as substrate by the method of Bessey-Lowry [2]. Enzyme activity was expressed in terms of p-nitrophenol units, one unit being defined as 1 µmol p-nitrophenol released per min per ml of the enzymatic solution. The protein content was determined routinely by reading the absorbance at 280 nm of the solution [3].

1) Electrophoretograms of the supernatant and the precipitate after WGA treatment

The electrophoretogram of the supernatant, the precipitate and the serum is shown in Fig. 1. The band of the supernatant showing enzymatic activity was observed at the “A” position. No bands were observed in the case of the precipitate. The precipitate was supposed to be composed of the high molecular complex (WGA-ALP complex), and it was therefore thought that the precipitate would not enter the gels.

A GlcNAc-epoxy-activated Sepharose 6B column was used to separate “ALP protein” from the “WGA-ALP complex”. After splitting the bond between WGA and “ALP protein”, the released “ALP protein” was electrophoresed to confirm whether it could enter the gels or not.

Fig. 1. Electrophoretogram of rat sALP before and after WGA treatment. Before: serum before WGA treatment, Supernatant: supernatant of serum after WGA treatment, Precipitate: precipitate after WGA treatment. The left gel was stained for ALP activity and the right gel for molecular weight (M.W.) standards was stained for protein. The direction of electrophoresis was toward the anode (bottom). 0 shows the sample application point.
2) GlcNAc-epoxy-activated Sepharose 6B column chromatography

The chromatographic profile is shown in Fig. 2. Three peaks of protein were eluted and ALP activity was detected in the second peak. The first peak was thought to be serum protein, which is not related to “ALP protein”. The third peak was thought to be WGA. As the concentration of GlcNAc solution was increased, the bond between “ALP protein” and WGA was cleaved and “ALP protein” was eluted prior to the elution of WGA.

3) Electrophoretogram of the “ALP protein”

The results of PAGE are shown in Fig. 3. In the lane corresponding to the supernatant, a band showing enzymatic activity was observed at the “A” position. In the lane corresponding to the second peak fraction obtained by chromatography (ALP protein), the band showing ALP activity was observed at the “B” position. Thus it was clarified that the released “ALP protein” could enter the gels and that it could be used as a marker.

The M.W. of ALP isoenzyme extracted from bone was 110 kDa and that of the “ALP protein”, described in this study, was 137 kDa (unpublished data). Righetti and Kaplan postulated that solubilization of membrane-bound tissue ALP in the laboratory with n-butanol was not physiological and undoubtedly cleaved ALP from the cell membrane at a different site than in vivo. As a result, different amounts of membrane lipid may be left attached to the protein moiety of ALP. Alternatively, sALP may be associated with other substances in blood.

Fig. 2. Elution profile of WGA-ALP complex from a GlcNAc Sepharose 6B column.

Fig. 3. Electrophoretogram of rat sALP isoenzymes fractionated from a GlcNAc Sepharose 6B column. Before: serum before WGA treatment, Supernatant: supernatant of serum after WGA treatment, ALP-Protein: the second peak in Fig. 2, which has ALP activity. The gel was stained for ALP activity. The direction of electrophoresis was toward the anode (bottom). 0 shows the sample application point.
The M.W. of the “ALP protein” is therefore thought to not correspond to that of ALP isoenzymes extracted from bone but, since WGA binds specifically to bone type sALP [1, 5], the “ALP protein” separated as described in this study is considered to be bone type sALP, and may serve as a marker of sALP isoenzymes subjected to PAGE.

References