SHORT COMMUNICATION

Sensitive Staining of Acidic Dentin Proteins with Electrophoresis

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Introduction

Dentin phosphoprotein (phosphophoryn, DPP) is a unique acidic protein in dentin. Except for type I collagen, DPP is the most abundant extracellular matrix protein in dentin. This protein is characterized by high contents of phosphoserine (45–50%) and aspartic acid (35–38%) (Butler *et al.*, 1995)¹⁾. Because of the high levels of aspartic acid and phosphoserine, this protein has polyanionic characteristics. The isoelectric point of this protein is 1.1 (Jonsson *et al.*, 1978)²⁾. DPP can bind a large number of calcium ions and affect *in vitro* mineralization. It forms insoluble aggregates in the presence of calcium ions (Kuboki *et al.*, 1979)³⁾. DPP is thought to play important roles in biomineralization.

A problem with the study of DPP is the difficulty in detection of this protein with high sensitivity. Because of DPP's highly acidic character, it is difficult to detect it with standard protein stainings, e.g. Coomassie Brilliant Blue or silver staining. A number of acidic proteins, such as DPP, are poorly stained on acrylamide gels using Coomassie blue or silver nitrate staining. Alcian blue or Stains-All have been used to stain acidic proteins (Sabsay *et al.*, 1991)⁴⁾, but both stainings have lower sensitivity than silver staining. Moller *et al.*, (1993) used combined Alcian Blue and Silver staining to stain proteoglycan sensitively (H.J. Moller *et al.*, 1993)⁵⁾. A combination of Stains-All and

Silver staining was also used to stain acidic proteins in bone (Goldberg *et al.*, 1997)⁶⁾.

In this study, we attempted to apply combined staining for acidic dentin proteins, especially DPP. We analyzed the dentin proteins extracted from a fragment of a single rat tooth.

Materials and Methods

1. Materials

Phosvitin was purchased from Taiyo Co. Incisors were obtained from a male Wister-King rat, 3 months old.

2. Demineralization and Extraction

Phosphophoryns were extracted and purified from rat incisors (Fujisawa *et al.*, 1984)⁷⁾. Briefly, the lower incisors of the rat were extracted (Wister-King rat, male, 3 months old), and enamel and soft tissue were removed mechanically. One incisor was divided into 3 fragments from the incisal to radicular sides. They were washed with TBS (50 mM Tris HCl, 150 mM NaCl pH 7.4, containing 1 mM benzyl sulfonyl fluoride, 5 mM benzamidine/HCl and 50 mM 6-amino hexanoic acid) for 1 h. After washing in this solution, the teeth were rinsed in distilled water and stored frozen until used.

The materials were broken into pieces in a stainless mortar and were extracted with 4 M guanidine/HCl, 50 mM Tris/HCl, pH 7.4, containing protease in-

hibitors (PI) for one week at 4°C. After the extraction, the mixture was centrifuged at 2,000 rpm for 20 min and the sediment was collected. The sediment was demineralized with 0.5 M EDTA, 50 mM Tris/HCl, pH 7.4, containing PI for one week at 4°C. Then the mixture was centrifuged (2,000 rpm, 20 min). After that, the supernatant was collected. The supernatant was lyophilized after dialysis against an excess of distilled water.

3. DEAE-Sephacel column Chromatography

The EDTA extracts were dissolved in 2 M urea 50 mM Tris/HCl, pH 8.2. Samples were load onto a DEAE-Sephacel column (2.0×23.5 cm) and eluted with an equilibration buffer (2 M urea 50 mM Tris/HCl, pH 8.2) at room temperature. A linear gradient of NaCl up to 1 M in the equilibration buffer was used to elute the DPP. Absorbance at 230 nm of the elute was monitored, and the phosphorus content in each fraction was determined. The fractions were dialyzed against distilled water prior to lyophilization.

4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoresis of proteins was done following protocols described by Laemmli (1970)⁸⁾. The gels (10% acrylamide, 0.375 M Tris/HCl pH 8.8, 0.1% SDS) were used for separation. Samples were dissolved in a sample buffer (20% glycerin, 1% SDS, 1% β -mercaptoethanol, 10 mM Tris-HCl, pH 6, 8) and were heated at 100°C for 3 min. The gels were electrophoresed at a constant current of 15 mA for 2 h.

5. Combined Alcian Blue and Silver Staining

After electrophoresis, the gels were fixed with 25% ethanol and 10% acetic acid for 2 h, changing the solution several times. Then the gels were stained with 0.125% Alcian blue in 25% ethanol and 10% acetic acid for 1 h. After staining, first the gels were destained with 25% ethanol and 10% acetic acid for 1 h, then with 10% ethanol and 5% acetic acid for 30 min, changing the solution several times. Then the gels were put in 5% glutardialdehyde for 20 min. After that the gels were rinsed with 10% ethanol and 5% acetic acid in water for 30 min, and then with water

for 12 min, changing the water twice. Finally, the gels were stained using a Silver Stain Kit (Wako, Osaka) according to the protocol.

Results and Discussion

We tried to stain phosvitin to examine the applicability of Alcian Blue-silver combined staining to phosphoproteins. Phosvitin is an acidic phosphoprotein in eggs and can not be stained by silver staining. 32—0.24 ng phosvitin was electrophoresed and the gels were stained with Alcian Blue alone or with combined staining (Fig. 1). No bands were observed in gel stained with Alcian Blue alone. With combined staining, a lower amount of phosvitin was detected. Bands of phosvitin were observed at 30 KDa. The lowest detection limit was 0.64 ng. The results indicate that combined staining is applicable to phosphoproteins. Sulfur-containing amino acids and basic amino acids are thought to be the reaction sites during silver staining. As phosvitin and phosphophoryn contain few of these amino acids, they can not be stained by silver staining. Alcian Blue is associated with acidic residues of the protein and may enhance nucleation of silver ions (Moller *et al.*, 1993)⁵⁾.

Phosphophoryn was purified partially by ion-exchange chromatography. The major phosphorus-containing peak was eluted at 0.3 M NaCl using DEAE-Sephacel chromatography (Fig. 2). This peak was collected and used as a phosphophoryn-enriched fraction in the following study. The phosphophoryn-enriched fraction was analyzed using combined staining (Fig. 3). 1 μ g—8 ng of the material was applied to the gels. With Alcian Blue staining alone, a band was observed only in the lane containing the highest amount of the material (1 μ g). Several bands were detected even in the lane containing the lowest amount of the material after combined staining. The lowest detection limit was less than 8 ng. Major bands were observed at 94, 90 and 80 KDa. Several investigators have reported the presence of multiple bands in the electrophoresis of rat phosphophoryn (Butler *et al.*, 1983⁹⁾, Rahima *et al.*, 1988¹⁰⁾). The presence of the three bands in the range of 90—94 KDa is consistent with these reports. Bands with lower molecular

Fig. 1 Staining of phosvitin in an electrophoretic gel with combined Alcian Blue and silver staining. Phosvitin (32, 16, 6.4, 3.2, 1.28, 0.64 ng for lanes 2–7, respectively) was applied to 10% acrylamide gel and electrophoresed. The gels were stained with Alcian Blue alone (A) or with the combined staining (B). Lane 1 : molecular weight marker.

masses, 80 and 40 KDa were not identified. They may be degradation products of the phosphophoryns. In this study, we detected as low as 8 ng of phosphophoryns. Combined staining was at least 120-times more sensitive than Alcian Blue staining. However, the sensitivity of the combined staining in the present study was not as sensitive as the original results reported by Moller *et al.* The difference in sensitivity can be attributed to differences in gel size. Moller *et al.* used microgel from Phastsystem, whereas we used conventional gel. Goldberg and Warner (1997) used Stains-All/silver staining and reported a lower detection limit of 0.25 ng for osteopontin. This detection limit is comparable to the detection limit for phosvitin in the present study.

As an application of combined staining, non-collagenous proteins obtained from different parts of a

Fig. 2 Anion exchange chromatography (DEAE-Sephacel column) of the EDTA extracts of rat incisors. Absorbance was read at 230 nm. A peak eluted at 0.3 M NaCl was collected as a phosphophoryn enriched fraction.

Fig. 3 Staining of a phosphophoryn enriched fraction with the combined Alcian Blue-silver staining. The phosphophoryn enriched fraction was prepared from EDTA extract of rat dentin by DEAE-Sephacel chromatography. The fraction (1 μ g, 0.2 μ g, 40 ng, 8 ng, for lanes 2–5) was applied to the 10% acrylamide gel. The gels were stained with Alcian Blue alone (A) or with the combined staining (B). Lane 1 : molecular weight marker.

Fig. 4 Staining of non-collagenous proteins (EDTA extracts) obtained from different parts of single rat tooth. Lane 2 : EDTA extract of undivided tooth (1 μ g). A rat incisor was divided into three parts : incisal, middle and radicular parts for lanes 3—5, (0.4 μ g) respectively. EDTA extract of the each parts was analyzed by gel electrophoresis using 10% acrylamide gel. The gels were stained with Alcian Blue alone (A) or with silver staining alone (B) or with the combined staining (C). Lane 1 : molecular weight marker.

single rat tooth were analyzed. Tooth fragments weighing as little as 46 mg were used as starting materials. The EDTA extracts from the fragments were analyzed by gel electrophoresis using combined staining (Fig. 4). No bands were observed in gel stained with Alcian Blue alone. Bands with molecular masses of 69 and 60 KDa were observed in gel stained

with silver staining alone. Several bands were observed in the range of 80—94 KDa in gel stained with combined staining, but not in a gel stained with silver staining alone. We think these bands correspond to bands of phosphophoryn and its derivatives from their staining properties.

There was a difference in the composition of the 80—94 KDa bands among the tooth fragments. The 94 KDa band was observed in every fragment. The incisal fragment contained more of the 90 KDa component than the radicular fragment. A lower molecular weight component (60 KDa) was measured in the incisal fragment. The difference indicates the maturation process in the matrix proteins, since the incisal fragment contains more processed proteins than the radicular fragment. Although these bands are not yet identified, investigators think that they include derivatives of the phosphophoryn molecule (Dimuzio *et al.*, 1985)¹¹⁾. Dimuzio *et al.* (1985) demonstrated in their biosynthetic study that an 80 KDa band appears at later stage. The 80 KDa component may be a degradation product of phosphophoryn. The turn over of phosphophoryn was demonstrated by a pulse-chase experiment (Dimuzio *et al.*, 1978)¹²⁾. A recent study using molecular cloning indicates that phosphophoryn may be derived from a larger-molecular-weight precursor dentin sialophosphoprotein, due to proteolytic processing (Macdougall *et al.*, 1997)¹³⁾. The multiple components of phosphophoryn may represent multiple cleavage sites in the precursor molecule and/or alternative splicing of the mRNA.

In conclusion, the staining method in the present study was sensitive enough to detect non collagenous proteins from a fragment of a single tooth, and enabled us to study the processing of dentin proteins without using radioisotopic labeling.

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