Laboratory Investigations

Characterization and Identification of a Human Dentin Phosphophoryn

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Abstract. The present study further characterizes an extract from immature, human tooth apicies from which an intact dentin phosphoprotein has been identified. Third molar apicies from developing roots were decalcified in 10% EDTA until Ca^{2+} was undetectable in the decalcifying solution. The crude extract was run on 7.5% SDS-PAGE and stained with ''Stains-All.'' Four distinct bands were found and the molecular weights were 140, 60, 50, and 34 k. When run on a SDS-PAGE under nonreducing conditions the 60, 50, and 34 k bands were absent. These results suggest that the lower molecular weight bands may be subunits of the larger protein. The extract was then further purified by adding $CaCl_2$ and $MgCl_2$ to precipitate the phosphoprotein. The precipitate was subjected to a DEAE-Sepharose CL6B column and eluted by 0–0.7 M NaCl gradient solution. The amino acid composition of the purified phosphoprotein was determined and the extract was found to be rich in serine and aspartic acid residues. The N-terminal peptide Asp-Asp-Pro was identified. The sequence of the three amino acids is identical to rat incisor phosphoprotein.

Key words: Human — Dentin — Phosphoprotein.

In all the mammalian species of dentin studied thus far, the major portion of the non-collagenous protein component has consisted of phosphoprotein [1]. Phosphoprotein has been shown to bind Ca^{2+} ions [2,3] and both catalyze hydroxyapatite crystal formation in highly supersaturated calcium phosphate solutions [4] and inhibit crystal growth when free in solution [5].

It has been reported that human dentin phosphoprotein from mature teeth appears to be degraded *in situ* [6], unlike rat dentin phosphoprotein, which when purified and subjected to SDS-PAGE, resolves as a distinct band [7]. Proteolytic degradation during the extraction procedures is one reported explanation for the lack of resolution of human dentin phosphoprotein [8]. Another is that human dentin phosphoprotein undergoes nonenzymatic degradation such as beta-elimination and aldol cleavage of serine, and racemization of some aspartyl residues during aging [9, 10]. Recently, McCurdy et al. [11] isolated a $Mr = 96$ k phosphoprotein from human, immature (open apex) tooth roots, which had a typical phosphoprotein amino acid profile characterized by increased levels of aspartic acid and serine. Using similar procedures, we have isolated and characterized a human dentinal phosphoprotein from immature tooth roots which has the biochemical profile of a phosphophoryn, a compositionally unique, highly phosphorylated protein [12], and compared our results to an extract from mature (closed apex) human teeth.

Materials and Methods

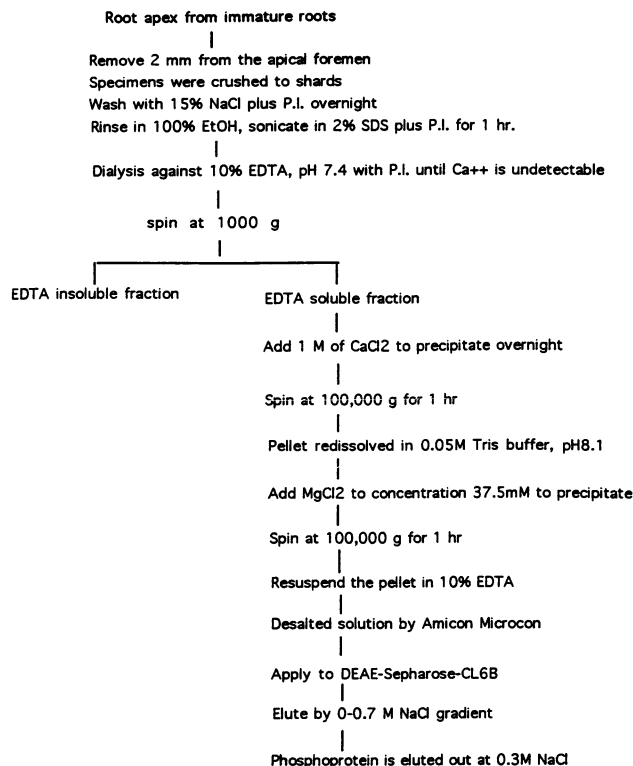### Extraction of Proteins from Immature Human Tooth Roots

Extracted, immature teeth with open root apicies were obtained from patients ranging in age from 8 to 16 years. Mature teeth with closed apicies were obtained from individuals over the age of 21. After extraction, all teeth were placed in 15% NaCl containing the following protease inhibitors: 2.5 mM benzamidine HCl, 50 mM ε-amino-n-caproic acid, 0.5 mM n-ethylmaleimide, and 0.3 mM phenylmethylsulphonylfluoride. The teeth were stored at −70°C before processing. All the tooth root shards were placed into a dialysis bag and demineralized in 10% EDTA plus protease inhibitors until no Ca^{2+} was detected in the EDTA solution. The EDTA soluble fraction was dialyzed against distilled water. Isolation and purification procedures of this crude extract are shown in Figure 1.

Polyacrylamide Gel Electrophoresis

Approximately 20 μg of the extract was loaded onto each lane of a 7.5% polyacrylamide resolving gel (0.1% SDS, with or without, 1% 2-mercaptoethanol) with a 4% stacking gel and electrophoresed at 150 volts for 40 minutes using a Mini-Protean II (BioRAD, Hercules, CA). The gels were stained with either Stains-All [13] or Coomassie blue.

Amino Acid, Sialic Acid, Carbohydrate Analysis, and Protein Sequencing

All protein samples were precleaned using Amicon Prospin column prior to analysis and sequencing. The analysis and sequencing

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Root apex from immature roots
|
Remove 2 mm from the apical foremen
Specimens were crushed to shards
Wash with 15% NaCl plus P.I. overnight
Rinse in 100% EtOH, sonicate in 2% SDS plus P.I. for 1 hr.
|
Dialysis against 10% EDTA, pH 7.4 with P.I. until Ca++ is undetectable
|
spin at 1000 g
|
┌──────────────────┴──────────────────┐
EDTA insoluble fraction   EDTA soluble fraction
                                         |
                                 Add 1 M of CaCl2 to precipitate overnight
                                         |
                                 Spin at 100,000 g for 1 hr
                                         |
                                 Pellet redissolved in 0.05M Tris buffer, pH8.1
                                         |
                                 Add MgCl2 to concentration 37.5mM to precipitate
                                         |
                                 Spin at 100,000 g for 1 hr
                                         |
                                 Resuspend the pellet in 10% EDTA
                                         |
                                 Desalted solution by Amicon Microcon
                                         |
                                 Apply to DEAE-Sepharose-CL6B
                                         |
                                 Elute by 0-0.7 M NaCl gradient
                                         |
                                 Phosphoprotein is eluted out at 0.3M NaCl

Fig. 1. Flow chart of human dentin phosphophoryn purification.

of phosphoprotein were conducted by the University of Michigan, Biomedical Core Facility. The methods are briefly described below.

Amino acid analysis was carried out using ABI model 420H amino acid analyzer by the phenylisothiocyanate method after automated acid hydrolysis. The free amino acids are automatically derivatized and resolved by on-line reverse-phase HPLC.

Carbohydrate analysis was carried out after hydrolysis in 4 N trifluoroacetic acid at an elevated temperature. The neutral and amino sugars released by this process were resolved by chromatography on a Dionex BioLC (Sunnyvale, CA), detected by pulsed amperometry at alkaline pH and compared to a standard carbohydrate mixture.

For analysis of sialic acid, 400 µl of 0.1 M HCl was added to the protein sample and heated for 1 hour at 80°C. The sample was dried and redissolved in 50 µl of distilled water and analyzed by Dionex BioLC anion exchange HPLC.

The protein sequence analysis by automated Edman degradation was carried out using standard procedures on a gas-phase sequencer (ABI model 470). The method involves reaction of the amino acid with the Edman reagent phenylisothiocyanate, at elevated pH (> pH 8), followed by cleavage of the derivatized amino-terminal residue catalyzed by a strong acid. The released compound is chemically converted to a more stable form and identified by its retention time on reverse-phase HPLC.

Phosphorus Measurement

Phosphorus was determined by the method of Ekman and Jager [14] using 2 M NaOH to hydrolyze the samples at 100°C for 15 minutes. The released phosphorus was quantified by the use of malachite green and phosphomolybdate.

Results

Purification of the Phosphoprotein

Four distinct Stains-All staining bands were seen after a

crude extract from immature tooth root was subjected to SDS-PAGE under reducing condition (Fig. 2A, lane 2). The highest molecular weight band was at 140 k and the lower molecular weight bands migrated at 60, 50, and 34 k. The 60, 50, and 34 k molecular weight bands were not visible after precipitating the crude extract with $CaCl_2$ and $MgCl_2$ (Fig. 2A, lane 3). This precipitate was then dissolved and further resolved on a DEAE-Sepharose CL6B column. A distinct 140 k protein band was detected on SDS-PAGE when stained with Stains-All (Fig. 2A, lane 4).

No distinct bands were seen when the crude extracts of human mature roots were electrophoresed on the polyacrylamide gel under reducing conditions and stained with Stains-All (Fig. 2A, lane 5). Precipitation of this extract with $CaCl_2$ and $MgCl_2$ and resuspension of the pellet prior to its exposure to the SDS polyacrylamide gel under reducing conditions also failed to produce any distinct Stains-All staining bands (Fig. 2A, lane 6).

Figure 2B shows the same protein samples electrophoresed under the same conditions as in Figure 2A but stained with Coomassie blue. In all the protein samples, the 140 k molecular weight band was not detected. The lower molecular weight protein bands, 60, 50, and 34 k were visible in the crude extract of the immature and mature tooth roots (Fig. 2B, lane 2 and 5, respectively).

As shown in Figure 3, when the same protein samples, except for the $CaCl_2$ and $MgCl_2$ precipitate of the mature tooth root crude extract, were electrophoresed under nonreducing conditions and stained with Stains-All, a single 140 k band was detected in lanes 2–4, and a much weaker staining 140 k band was seen in lane 5.

The elution profile of the $CaCl_2$ and $MgCl_2$ precipitate, when subjected to DEAE-Sepharose column, showed one major peak at a NaCl concentration of approximately 0.35 M (Fig. 4). The peak has a very high phosphorus content of approximately 5.2% by weight.

Characterization of the Phosphoprotein

The composition of the amino acid profiles for the extracts from immature human tooth roots is shown in Table 1. Column 1 identifies the amino acids and columns 2-4 contain their actual amounts as a percentage of the total number of amino acid residues. When columns 3 and 4, which contain the amino acid composition of the purified protein extracts from immature tooth roots, were compared with column 2, the crude extract amino acid composition, an increase in the percentage of aspartic acid and serine residues of approximately 15% and 10%, respectively, was noted. The carbohydrate composition of the final purified phosphoprotein from immature tooth roots is also shown in Table 1. The only carbohydrate found in this extract was glucosamine.

The sequencing of the purified phosphoprotein (Mr = 140 k) resulted in an amino acid sequence from the N-terminal of Asp-Asp-Pro being obtained (Table 2). We also purified phosphoprotein from rat using the same method as for human and obtained a 12 amino acid sequence of Asp-Asp-Pro-Asn(?)-Ser(?)-?-Asp-Glu-Ser-Asn-Gly-?-Asp-Asp(?) from the N-terminal (Table 2).

Discussion

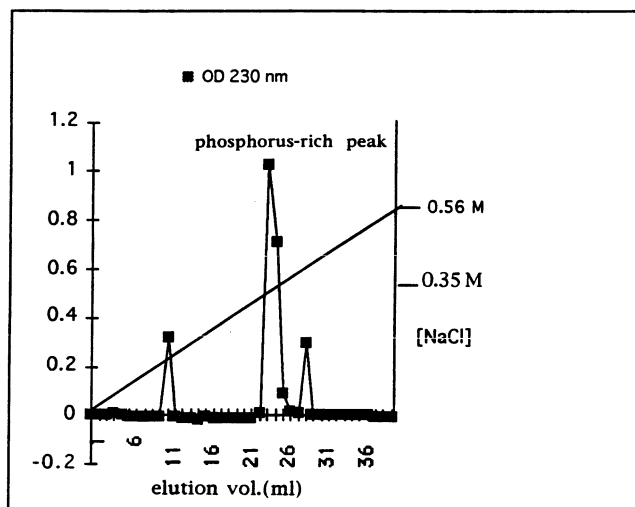A phosphorus-rich protein was isolated from human imma-

A
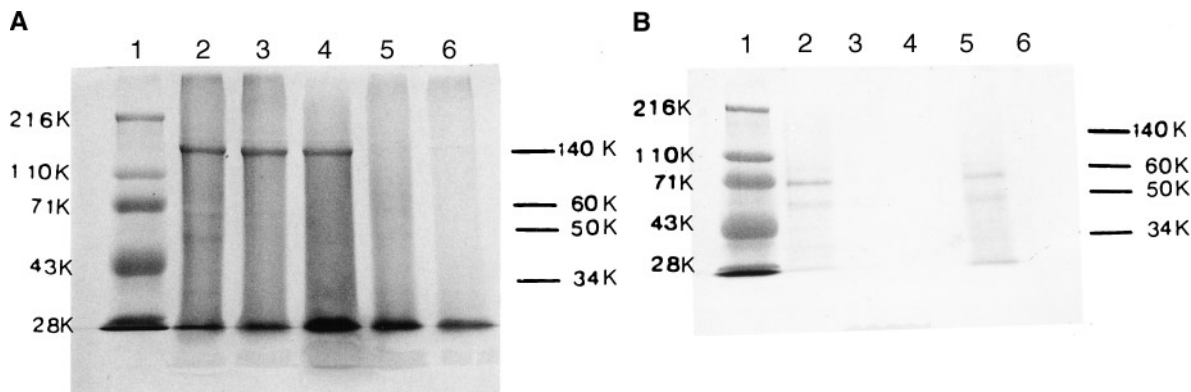
Fig. 2(A) SDS PAGE (7.5%)-reducing gel stained with Stains-All. Lane 1: protein marker; Lane 2: crude extract of human immature tooth root dentin; Lane 3: $CaCl_2$ and $MgCl_2$ precipitated extract of human immature tooth root dentin; Lane 4: $CaCl_2$ and $MgCl_2$ precipitated and anion-exchange column-purified protein

B

of human immature root dentin; Lane 5: crude extract of human mature tooth root dentin; Lane 6: $CaCl_2$ and $MgCl_2$ precipitated extract of human mature root dentin. (Lane numbers in Fig. 2A and 2B correspond to the same extracts for both figures.) **(B)** SDS-PAGE (7.5%)-reducing gels stained with Coomassie blue.
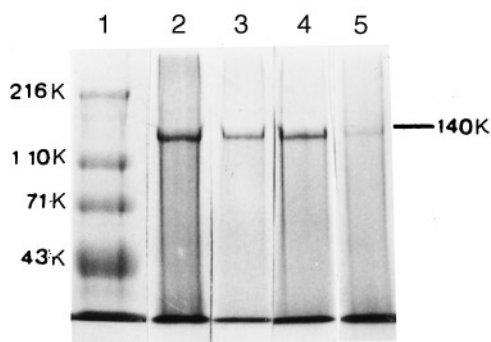
Fig. 3. Stains-All stained 7.5% SDS-PAGE, nonreducing gel. Lane 1: protein marker; Lane 2: crude extract of human immature tooth root dentin; Lane 3: $CaCl_2$ and $MgCl_2$ precipitated extract of human immature tooth root dentin; Lane 4: $CaCl_2 + MgCl_2$ precipitated and anion exchange column purified protein of human immature root dentin; Lane 5: crude extract of human mature tooth root dentin.

Fig. 4. DEAE-Sepharose column elution profile of $CaCl_2 + MgCl_2$-precipitated extract of human tooth root dentin.

ture tooth roots, which when run on 7.5% SDS polyacrylamide reducing gel, gave a distinct Stains-All staining band at 140 k. This band is thought to be similar to the 96 k protein band which was resolved on a 12% SDS polyacrylamide gel from an extract of human teeth and described by McCurdy et al. [11] and MacDougall et al. [15]. Three other bands of lower molecular weight also stained with Stains-All, but were not visible on a nonreducing gel. This suggests that these bands may be subunits of the larger, intact protein. We were also unable to isolate an intact phosphoprotein from mature root dentin. These results are consistent with those reported by Masters [10] which showed that phosphoprotein from human dentin may undergo degradation during its maturation. The presence of a weakly staining band at a molecular weight of 140 k on nonreducing gel for mature tissue was unexpected. However, although we were using closed apex (mature) root extracts for this nonreducing gel (Fig. 3, Lane 5), these specimens were taken from a large number of teeth from people of different ages. Therefore, Figure 3, Lane 5 reflects the staining of an extract from a mixture of mature and immature tissue which may account for the weakly staining band seen on this gel.

The amino acid composition of the 140 k molecular weight protein band was consistent with that described for other mammalian dentin phosphoproteins [7, 16]. The human 140 k protein contained serine (phosphoserine) and aspartic acid residues which comprised approximately 76% (observed data) of the amino acid composition, and therefore defined this protein as a phosphophoryn [17].

This human phosphophoryn, like bovine phosphophoryn, would appear to be a single protein (Fig. 2A) whereas multiple forms phosphoproteins have been isolated from rat incisors [7]. Takagi et al. [18] reported that there were differences in the amount of phosphoprotein extracted from the crown and root of human teeth, and these phosphoproteins may also show differences in their biochemical composition. Therefore, had we used a mixed sample of crown and root dentin we may have detected phosphoproteins of different molecular weights.

We believe that this is the first reported isolation, identification, and partial characterization of human root dentinal phosphophoryn and it would appear that developing

Table 1. Amino acid and carbohydrate compositions of phosphoprotein from immature dentin (at different stage of purification)

Amino acid[a]	crude extract (immature)	$CaCl_2$ + $MgCl_2$ ppt (immature)	$CaCl_2$ + $MgCl_2$ and column purified (immature)
Asp	26.8	43.0	41.4
*+Ser	22.8	29.2	35
His	1.3	0.7	0
Thr	3.5	1.5	2.1
Pro	4.3	2.2	2.8
Val	2.8	1.7	2.1
Cys	0	0	0
Leu	3.8	2.2	0.7
Lys	5.0	2.2	2.1
Glu	9.3	5.8	3.6
Gly	8.1	5.1	5.0
Arg	3.0	0.7	0.7
Ala	5.0	4.4	2.8
Tyr	1.1	0.7	0.7
Met	0	0	0
Ile	1.6	0.7	0.7
Phe	1.6	0.7	0
Trp	0	0	0
Carbohydrate			
Sialic acid	————	not detected	not detected
Galatosamine	————	not detected	not detected
Glucosamine	————	present	present

[a] Amino acid residues are a percentage of total composition
* Serine includes phosphoserine
+ Above value is the observed value without any calculation of degraded phosphoserine + serine

Table 2. Comparison of amino acid residue N-terminal sequences by different investigators

Cycle	Human DPP	Rat DPP	Rat DPP [7]	Rat DPP [15]
1	Asp	Asp	Asp	Asp
2	Asp	Asp	Asp	Asp
3	Pro	Pro	Pro, Asp	Pro
4		Asn(?)	Asn	Asn
5		Ser(?)	Asp(Ser?)	Asp
6		?	Asp(Ser?)	Asp
7		Asp		Asp
8		Glu		Glu
9		Ser		
10		Asn		
11		Gly		
12		?		
13		Asp		
14		Asp(?)		

80% (observed data) of serine (phosphoserine) and aspartic acid residues and a 12 amino acid sequence similar to that previously reported [7]. Using the same techniques, we have been able to repeatedly obtain a three N-terminal amino acid sequence of a human dentinal phosphophoryn. This amino acid sequence is identical to that described by Butler et al. [7] and MacDougall et al. [15] for rat phosphoprotein. Although only a small number of amino acid sequences of phosphophoryn have been compared, the similarity of amino acid sequences of phosphophoryn between different species indicates that it may be highly conserved. Further work is continuing in the isolation, purification, and sequencing of this protein to identify a larger amino acid sequence.

tooth roots must be used to obtain an intact protein specimen. However, the paucity of tissue represents a significant problem. Five grams of apical tissue, representing approximately 100 teeth, yields only 10 mg of total protein. Further purification results in a loss of about 90% of protein. Thus, in preliminary studies, we have used rat dentin extract to optimize our extraction and purification procedures. In unpublished studies, using these optimized techniques, we have isolated a rat phosphophoryn containing more than

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