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Protocols for Studying Formation and Mineralization of Dental Tissues In Vivo: Extraction Protocol for Isolating Dentin Matrix Proteins from Developing Teeth

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Abstract

The organic material in developing dentin is 90% type I collagen and 10% non-collagenous proteins. The key to understanding dentin biomineralization is to study how these proteins collectively precipitate and organize hydroxyapatite crystals. The first step in characterizing the proteins within a mineralizing matrix is to efficiently extract and isolate the essential molecular participants and elucidate their structural and biochemical properties. In this study, we expanded previous approaches to develop an improved strategy for the extraction of extracellular matrix proteins from the dentin of developing teeth. Proteins in dentin powder were sequentially extracted in the order Tris-guanidine buffer, HCl-formic acid solution, acetic acid-NaCl solution, Tris-NaCl buffer, and a second Tris-guanidine buffer. Individual fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by gelatin or casein zymography, and by Western blot analysis using dentin sialoprotein (DSP)- or dentin glycoprotein (DGP)-specific antibodies. This approach was used to purify assorted porcine dentin non-collagenous proteins.

Keywords

Dentin; Teeth; Non-collagenous protein; Extraction; DSPP; Phosphoryn

1 Introduction

Dentin is the mineralized tissue comprising the body of a tooth. It protects the soft tissue pulp within and supports the overlying enamel and cementum. On a weight basis, dentin is about 70% mineral, 20% organic matrix, and 10% water. Dentin forms in a defined extracellular space by matrix-mediated biomineralization. Secreted proteins regulate and control the mineralization process. With more than 40 years of experience isolating and

characterizing dentin proteins, many of the extraction and isolation procedures have been optimized and standardized. Historically, a dissociative extraction procedure using guanidine and the chelating agent EDTA (ethylenediaminetetraacetic acid) was used for the extraction of proteins from dentin matrix [1–10], followed by phosphoprotein-specific calcium precipitation [11], which efficiently isolated dentin phosphoprotein (DPP), the highly acidic C-terminal domain of dentin sialophosphoprotein (DSPP) [12]. In the early 1990s, the guanidine-EDTA-based method was also used for the fractionation of dentin non-collagenous proteins other than phosphoprotein such as dentin sialoprotein (DSP) [13–16]. We used this method to isolate and characterize DSP [17] and dentin glycoprotein (DGP) [18] from developing porcine teeth. Shortly thereafter, we developed a sequential extraction method using successive extraction solutions, instead of the guanidine-EDTA-based method [19]. The serial extraction method was later improved using a rapid demineralization step, which replaced the 6-day acetic acid extraction with mixture of hydrochloric acid and formic acid extraction for 1 day. The rapid demineralization step greatly reduced postmortem proteolysis and allowed us to fractionate intact dentin sialophosphoprotein (DSPP), the parent protein of DSP, DGP, and DPP [20]. In this chapter, we explain the dentin serial extraction protocol and subsequent methods for chromatographic separation of the major non-collagenous dentin matrix proteins and visualize the results of these separations using SDS-PAGE, zymography, and Western blot analyses.

2 Materials

2.1 Developing Porcine Tooth

Tooth germs of permanent second molars were surgically extracted using a hammer and chisel to remove them from the mandibles of 5-month-old pigs acquired from the Meat Market of the Metropolitan Central Wholesale Market (Shinagawa, Tokyo, Japan) (Fig. 1a). After removing the surrounding soft tissues and inner pulp tissues with forceps, the tooth germs were washed in cold saline and wiped carefully with a Kimwipe. The molars obtained from these 5-month-old pigs were in the developmental stage of advanced crown formation but prior to the onset of root formation (Fig. 1b).

2.2 Extracting Solutions for Sequential Extraction (See Notes 1 and 2)

1. 50 mM Tris-HCl/4 M guanidine (TG) buffer (pH 7.4): Weigh 6.057 g Tris and 382.12 g guanidine hydrochloride in a glass beaker, and add purified water to a volume of 900 mL. Mix and adjust pH with 6 N HCl to pH 7.4. Make up to 1 L with purified water. Store at 4 °C. Remove the contaminant in buffer solution with a glass vacuum filter.
2. 0.17 N HCl and 0.95% formic acid (HF) solution: Add 42.4 mL of concentrated HCl and 80 mL of 95% formic acid in a 10 L plastic pail. Make up to 8 L with distilled water. Store at 4 °C.

¹-Except for the HF solution, prepare all solutions using ultra-pure water (prepared by purifying deionized water), to attain a resistance of 18 MΩ-cm at room temperature.

²-The TG, AN, and TN solutions contain 0.6 g benzamidine (5 mM as final concentration), 1 mL of 1 M phenylmethylsulfonyl fluoride (PMSF) (1 mM as final concentration), and 1 mL of 1 M 1,10-phenanthroline (1 mM as final concentration).

3. 0.5 M acidic acid/2 M NaCl (AN) solution: Weigh 116.88 g NaCl in a 1 L graduated cylinder, and add purified water to a volume of 900 mL. After the dissolution, add 29 mL of acetic acid, and make up to 1 L with purified water. Store at 4 °C.
4. 50 mM Tris-HCl/2 M NaCl (TN) buffer (pH 7.4): Weigh 6.057 g Tris and 116.88 g NaCl in a glass beaker, and add purified water to a volume of 900 mL. Mix and adjust pH with 6 N HCl to pH 7.4. Make up to 1 L with purified water. Store at 4 °C. Remove the contaminant in buffer solution with a glass vacuum filter.

2.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Novex 4–20% Tris-Glycine Mini Protein Gel (1.0 mm, 12-well).
2. Novex 10% Zymogram (Gelatin) Protein Gel (1.0 mm, 10-well)
3. Novex 12% Zymogram (Casein) Protein Gel (1.0 mm, 10-well)

2.3.1 SDS-PAGE and Zymogram Gels (See Note 3)

1. NuPAGE 4–12% Bis-Tris Protein Gel (1.0 mm, 12-well)

2.3.2 Running Buffer (See Note 3)

1. Tris-Glycine SDS running buffer: Add 100 mL of Novex Tris-Glycine SDS running buffer (10×) in a 1 L graduated cylinder, and make up to 1 L with purified water. Store at room temperature.
2. MES SDS running buffer: Add 50 mL of NuPAGE MES SDS running buffer (20×) in a 1 L graduated cylinder, and make up to 1 L with purified water. Store at room temperature.
3. MOPS SDS running buffer: Add 50 mL of NuPAGE MOPSSDS running buffer (20×) in a 1 L graduated cylinder, and make up to 1 L with purified water. Store at room temperature.

2.3.3 Sample Buffer (See Note 3)

1. NuPAGE LDS Sample Buffer (4×).
2. Novex Tris-Glycine SDS Sample Buffer (2×).

2.3.4 Coomassie Brilliant Blue (CBB) Solution

1. SimplyBlue SafeStain (Invitrogen-Thermo Fisher Scientific) for SDS-PAGE.
2. Coomassie Brilliant Blue (CBB) solution for Zymogram: Weigh 2.5 g of CBB R-250 (BIO-RAD, Hercules, CA, USA) in a glass beaker. Add 500 mL of methanol, 100 mL of acetic acid, and 400 mL of purified water, and mix well. Filter into a plastic reagent bottle. Store at room temperature.

³. We purchased all SDS-PAGE and Zymogram Gels, running buffers, and sample solutions from Invitrogen-Thermo Fisher Scientific.

2.3.5 Stains-All Solution

1. Stock solution: Weigh 0.1 g Stains-All (Sigma-Aldrich, St. Louis, MO, USA) in 50 mL centrifuge tube, and dissolve with 20 mL of formamide. Store at room temperature protected from light.
2. Staining solution: Mix 135 mL of 30 mM Tris-HCl buffer (pH 8.8), 15 mL of formamide, 50 mL of methanol, and 1 mL of Stains-All stock solution in 200 mL graduated cylinder (*see Note 4*).

2.3.6 Destaining Solution for Zymogram

1. Add 1.5 L of methanol, 300 mL of acetic acid, and 1.2 L of purified water in a plastic reagent bottle.
2. Store at room temperature.

2.4 Western Blotting (See Note 5)

1. Tris-Glycine transfer buffer: Add 40 mL of Novex Tris-Glycine transfer buffer (25×) in a 1 L graduated cylinder, and make up to 1 L with purified water. Store at room temperature.
2. NuPAGE transfer buffer: Add 50 mL of NuPAGE transfer buffer (20×) in a 1 L graduated cylinder, and make up to 1 L with purified water. Store at room temperature.
3. Invitrogen PVDF/Filter Paper Sandwich (0.45 m pore size, 8.3 cm × 7.3 cm).
4. Western Blotting Filter Paper, extra thick (20 cm × 20 cm).
5. Tris-buffered saline (TBS)-Tween (TBST) solution: Add 200 mL of TBS (10×) and 20 mL of 10% Tween 20 in a graduated cylinder. Make up to 2 L with purified water. Store at room temperature.
6. Blocking solution: Weigh 5 g of nonfat dry milk, and dissolve it with 100 mL in TBST.

3 Methods

3.1 Preparation of Dentin Powder

1. Scrape off the enamel layer with a curette from second molars (Fig. 1b).
2. Pulverize the remaining hard tissue to powder using a jaw crusher (Fig. 1c) (*see Note 6*).
3. Yields approximately 20 g of tooth powder from 16 molars (8 pigs) (Fig. 1d).

⁴. Prepare the staining solution the third end rinsed 5 min before.

⁵. We purchased all transfer buffers and filter paper from Invitrogen-Thermo Fisher Scientific and TBS (10×), 10% Tween 20, and nonfat dry milk from BIO-RAD.

⁶. Mixer Mill MM400 (Retsch, Newtown, PA, USA).

3.2 Sequential Extraction of Proteins from Tooth Powder (Fig. 2)

Carry out all procedures under ice-cold conditions unless otherwise specified.

3.2.1 First Tris-Guanidine Extract (G1 Extraction) (See Note 7)

1. Add 10 g of tooth powder to 250 mL for the plastic centrifuge tube.
2. Suspend with 100 mL of TG buffer.
3. Homogenize using a Polytron homogenizer for 1 min at half speed.
4. Centrifuge for 10 min at $15,900 \times g$ at 4 °C.
5. Collect the supernatant (G1 extract) in a glass beaker.
6. Repeat **steps 2–5** two more times for insoluble material.
7. Transfer G1 extract to a Spectra/Por 3 membrane.
8. Dialyze against 16 L of distilled water for 3 days at 4 °C while exchanging daily.
9. Separate the soluble (G1S extract) and insoluble (G1P extract) fractions by centrifugation for 10 min at $15,900 \times g$ at 4 °C.
10. Lyophilize both G1S and G1P extracts.

3.2.2 Demineralization (HF Extraction) (See Note 8)

1. Pack the Tris-guanidine insoluble material in a dialysis bag using a spatula from the plastic centrifuge tube as much as possible.
2. Suspend the remaining Tris-guanidine insoluble material in the plastic centrifuge tube with 5 mL of HF solution.
3. Pipetting it several times and transfer to a dialysis bag (*see Note 9*).
4. Repeat **step 3** until the residue disappears as much as possible (*see Note 10*).
5. Dialyze against 8 L of HF solution for 1 day at 4 °C.
6. Transfer the dialysis bag contents to the centrifuge tube.
7. Centrifuge for 10 min at $15,900 \times g$ at 4 °C.
8. Collect the supernatant (HF extract) in an Erlenmeyer flask (*see Note 11*).
9. Transfer HF extract to a Spectra/Por 3 membrane.
10. Dialyze against 16 L of distilled water for 3 days at 4 °C while exchanging daily.
11. Lyophilize HF extract.

⁷The purpose of the G1 extraction is to remove remaining enamel proteins (mostly amelogenins) and blood and tissue components.

⁸The purpose of the HF extraction is to demineralize and there by extract proteins having an affinity for hydroxyapatite.

⁹Spectra/Por 3 Dialysis Membrane (nominal flat width, 54 mm; diameter, 34 mm) (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA).

¹⁰Final volume in dialysis bag should be less than 50 mL.

¹¹As the insoluble materials sometimes become smooth particles after the demineralization, we mainly collect HF, AN, TN, and G2 extracts through a glass vacuum filter.

3.2.3 Acetic Acid-NaCl Extract (AN Extraction) (See Note 12)

1. Pack the HF insoluble material in the plastic centrifuge tube using a spatula from the surface of glass vacuum filter as much as possible.
2. Suspend with 100 mL of AN solution.
3. Homogenize using a Polytron homogenizer for 1 min at half speed.
4. Centrifuge for 10 min at $15,900 \times g$ at 4 °C.
5. Collect the supernatant (AN extract) in the Erlenmeyer flask (*see Note 11*).
6. Repeat **steps 2–5** two more times for insoluble material.
7. Transfer AN extract to a Spectra/Por 3 membrane.
8. Dialyze against 16 L of distilled water for 3 days at 4 °C while exchanging daily.
9. Lyophilize AN extract.

3.2.4 Tris-NaCl Extract (TN Extraction) (See Note 13)

1. Pack the AN insoluble material in the plastic centrifuge tube using a spatula from the surface of glass vacuum filter as much as possible.
2. Suspend with 100 mL of TN buffer.
3. Homogenize using a Polytron homogenizer for 1 min at half speed.
4. Centrifuge for 10 min at $15,900 \times g$ at 4 °C.
5. Collect the supernatant (TN extract) in the Erlenmeyer flask (*see Note 11*).
6. Repeat **steps 2–5** two more times for insoluble material.
7. Transfer TN extract to a Spectra/Por 3 membrane.
8. Dialyze against 16 L of distilled water for 3 days at 4 °C while exchanging daily.
9. Lyophilize TN extract.

3.2.5 Second Tris-Guanidine Extract (G2 Extraction) (See Note 14)

1. Pack the TN insoluble material in the plastic centrifuge tube using a spatula from the surface of glass vacuum filter as much as possible.
2. Suspend with 100 mL of TG buffer.
3. Homogenize using a Polytron homogenizer for 1 min at half speed.
4. Centrifuge for 10 min at $15,900 \times g$ at 4 °C.
5. Collect the supernatant (G2 extract) in the Erlenmeyer flask (*see Note 11*).
6. Repeat **steps 2–5** two more times for insoluble material.

¹²The purpose of the AN extraction is to extract proteins ionically bound to collagen.

¹³The purpose of the TN extraction is also to extract proteins ionically bound to collagen and to neutralize for next guanidine step.

¹⁴The purpose of the G2 extraction is to extract proteins binding to the collagen.

7. Transfer G2 extract to a Spectra/Por 3 membrane.
8. Dialyze against 16 L of distilled water for 3 days at 4 °C while exchanging daily.
9. Lyophilize G2 extract.

3.2.6 Preparation of Residue Insoluble Material (RIM) (See Note 15)

1. Pack the G2 insoluble material in the plastic centrifuge tube using a spatula from the surface of glass vacuum filter as much as possible.
2. Suspend with 100 mL of purified water.
3. Centrifuge for 10 min at $15,900 \times g$ at 4 °C.
4. Discard the supernatant.
5. Repeat **steps 2–4** two more times for insoluble material.
6. Lyophilize insoluble material.

3.3 SDS-PAGE

Our electrophoresis has been carried out using NuPAGE Bis-Tris Mini Gels or Novex Pre-Cast Gels (Tris-Glycine SDS and Zymogram Gels) and performed in accordance with the general information and protocols for NuPAGE Bis-Tris Mini Gels or Novex Pre-Cast Gels [21] (*see Note 16*). Here, we only show the preparation of dentin sample for SDS-PAGE and results of SDS-PAGE, Western blot, and Zymogram (Fig. 3).

3.3.1 Sample Preparation

1. Weigh 300–400 µg of each lyophilized extract in an Eppendorf tube, and add purified water as it becomes the concentration of 2 µg/µL.
2. Transfer 75 µL or 50 µL to another Eppendorf tube, and dissolve with 25 µL of NuPAGE LDS Sample Buffer (4×) or 50 µL of Novex Tris-Glycine SDS Sample Buffer (2×).
3. Load 6–7 µL (9–10.5 µg) on the gels.
4. Run electrophoresis in accordance with the general information and protocols.

3.3.2 CBB Staining

1. Rinse the gel with 100 mL of purified water for 5 min at three times.
2. Stain the gel with 50 mL of SimplyBlue SafeStain for 1 h at room temperature.
3. Wash the gel with 100 mL of purified water for at least 3 h.

3.3.3 Stains-All Staining

1. Rinse the gel with 200 mL of 25% methanol for 30 min at three times.

¹⁵Because the main component in RIM is an insoluble collagen, this step is optional.

¹⁶Refer to: https://tools.thermofisher.com/content/sfs/manuals/MAN0007891_NuPAGE_BisTris_MiniGels.pdf

2. Stain the gel with 200 mL of Stains-All solution overnight in the dark.
3. Destain the gel with 200 mL of 25% methanol for at least 1 h.

3.3.4 Zymography

1. Rinse the Zymogram Gel with 100 mL of 2.5% Triton X 100 for 30 min two times.
2. Incubate the gel with 200 mL of 50 mM Tris-HCl buffer (pH 7.4) overnight.
3. Stain the gel with 100 mL of CBB for zymography at room temperature for 30 min.
4. Destain the gel with 100 mL of purified water for at least 3 h (*see Note 17*).

3.4 Western Blotting

1. Electrotransfer the gel onto the PVDF membrane which has been carried out in accordance with protocols of overview for Western blotting (Thermo Fisher Scientific) (*see Note 18*).
2. Block the membrane with 5% skim milk in TBST for at least 1 h at room temperature (RT) (*see Note 19*).
3. Add primary antibody in TBST containing 5% bovine serum albumin (BSA).
4. Incubate the membrane overnight in 4 °C on a shaker.
5. Wash the membrane with TBST for 10 min three times on a shaker at RT.
6. Add secondary antibody in TBST containing 5% BSA for 1 h at RT.
7. Wash the membrane with TBST for 10 min three times on a shaker at room temperature.
8. During the washing, prepare ECL solution following the proportion of solutions A and B provided by the manufacture (*see Note 20*).
9. Incubate the membrane for 5 min at RT.
10. Visualize the result in the dark room (*see Note 21*).

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¹⁷·We usually use five to six about the blocked cube sponge (each size: 2 cm × 2 cm × 2 cm) to enhance the adsorption of the CBB dye.

¹⁸·Refer to: <https://www.thermofisher.com/jp/ja/home/references/protocols/proteins-expression-isolation-and-analysis/western-blot-protocol/electrophoresis-and-blotting.html#prot1>

¹⁹·We sometimes perform the blocking step for overnight in 4 °C on a shaker.

²⁰·We use Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Uppsala, Sweden).

²¹·We used Amersham Imager 600 (GE Healthcare Life Sciences).

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Fig. 1.

Preparation of dentin powder from developing porcine tooth. (a) Tooth germs of permanent second molars in the mandibles of a 5-month-old pig. (b) Surgically extracted second molar is in the crown formation stage and has a mesial-distal dimension of about 2 cm. (c) The second molar after scraping off the enamel layer. (d) 20 g of dentin powder in a container obtained by pulverizing 16 second molars

Fig. 2.

Flowchart showing the procedures used to produce the primary extracts for the characterization of proteins from dentin powder. *Sup* supernatant, *Ppt* precipitate, ext extract, *RIS* residue insoluble materials

Fig. 3.

Primary dentin extracts. The eight primary dentin extracts are G1S, G1P, HF, AN, TN, G2S, G2P, and RIS. (a, b) Porcine dentin powder extracts analyzed by SDS-PAGE stained with CBB and Stains-All. (c) Western blot of SDS-PAGE using the DSP antibody. (d, e) Gelatin and casein Zymogram of dentin extracts (*see Note 22*). Number indicates protein bands which were identified in dentin extracts. 1 remaining enamel proteins, 2 acid soluble collagen, 3 osteonectin (SPARC), 4 dentin glycoprotein (DGP), 5 osteocalcin (BGP), 6 high molecular weight dentin sialoprotein (HMW-DSP), 7 dentin phosphoprotein (DPP), 8 albumin, 9 insoluble collagen, 10 low molecular weight DSP (LMW-DSP), 11 matrix metalloproteinase 2 (MMP-2), 12 kallikrein 4, 13 MMP-20

²²The intact DSPP is extracted in G2P fraction, but it is not detected by Western blot at this point because of a large amount of collagen degradation products. We further fractionate the intact DSPP by ion-exchange chromatography and reverse-phase high-performance liquid chromatography.