DENTIN SIALOPHOSPHOPROTEIN IS PROCESSED BY MMP-2 AND MMP-20 IN VITRO AND IN VIVO

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Dentin sialophosphoprotein (DSP) is a major secretory product of odontoblasts, and is critical for proper tooth dentin formation. During dentinogenesis DSP is proteolytically cleaved into smaller subunits. These cleavages are proposed activation steps and failure to make these cleavages is a potential cause of developmental tooth defects. We tested the hypothesis that dentin-resident matrix metalloproteinases catalyze the cleavages that process DSP. We defined the exact DSP cleavages that are catalyzed by proteases during crown formation, by isolating DSP-derived proteins from developing porcine molars and characterizing their N-terminal sequences and apparent size on SDS-PAGE and Western blots. The in vivo DSP cleavage sites were on the N-terminal sides of Thr1200, Ser1330, Val153, Leu369, Ile362, Ser377, Ser408, and Asp458. The initial DSP cleavage is between dentin glycoprotein (DGP) and dentin phosphoprotein (DPP), generating DSP-DGP and DPP. Gelatin and casein zymograms identified MMP-2, MMP-20, and KLK4 in the dentin extracts. MMP-2 and MMP-20 were purified from over 150 g of porcine dentin powder and incubated with DSP-DGP and DPP. These enzymes show no activity in further cleaving DPP. MMP-20 cleaves DSP-DGP to generate dentin sialoprotein (DSP) and DGP. MMP-20 also cleaves DSP at multiple sites, releasing N-terminal DSP cleavage products ranging from 25- to 38-kDa. MMP-2 makes multiple cleavages near the DSP C-terminus, releasing larger forms of DGP, or “extended DGP”. Exact correspondence between DSP cleavage sites that occur in vivo and those generated in vitro demonstrate that MMP-2 and MMP-20 process DSP into smaller subunits in the dentin matrix during odontogenesis.

Dentin sialophosphoprotein (DSP) is a multi-domain extracellular matrix protein that is critical for proper dentin formation. The major DSP domains are dentin sialoprotein (DSP), dentin glycoprotein (DGP), and dentin phosphoprotein (DPP) (1-3). In humans, nine disease-causing mutations in the DSP gene on chromosome 4q21.3 have been reported in kindreds with isolated inherited dentin defects (4-10). No other genes have been implicated in their etiology by mutation analyses. DSP mutations result in a variety of dental phenotypes, including dentin dysplasia (DD) type II, and dentinogenesis imperfecta (DGI) types II and III. In the mouse, targeted knockout of the Dsp gene (Dsp<sup>−/−</sup>) resulted in tooth defects resembling DGI type III (11). DSP is expressed in other tissues besides dentin, such as in bone (12), but the protein levels in the secondary locations are hundreds of times lower than in dentin. Outside of a poorly understood association with progressive high
frequency sensorineural hearing loss, DSPP mutations result in defects limited to the teeth. Perhaps because DSPP functions are so specialized, relatively little research has been focused on it, and many of its basic structural features are still unknown.

DSPP itself has never been isolated or detected in dentin extracts. DSPP was discovered through its proteolytic cleavage products, starting with dentin sialophosphoprotein (DPP) (13). DPP is a highly acidic phosphoprotein with an isoelectric pH of 1.1 (14). Besides its distinctive amino acid composition, dominated by serine and aspartic acid (15), DPP has a conserved N-terminal sequence of Asp-Asp-Pro-Asn (1). DPP is difficult to study at the protein level because it is resistant to digestion by proteases such as trypsin (16). Cloning and characterization of the first DPP cDNA transcript revealed the extreme redundancy of its DNA and deduced amino acid sequences (17). Surprisingly, the cloned DPP transcript also encoded dentin sialoprotein (DSP), another major non-collagenous protein in dentin (18,19). DSP is a highly glycosylated proteoglycan (19-21). Demonstration of a continuous open reading frame between the DSP and DPP coding regions disclosed that DSP and DPP are cleavage products of a larger protein, called dentin sialophosphoprotein (DSPP) (22). Dentin glycoprotein (DGP) is the most recent and smallest DSPP-derived protein to be characterized (3). DSP is at the N-terminus of DSPP, DGP is in the middle, and DPP is at the C-terminus. The finding that proteolysis is necessary to generate DSP and DPP fostered the hypotheses that proteolytic cleavages are necessary activation steps, and that specific proteases might be involved (23,24).

To gain insight into the structures and functions of DSPP-derived proteins, we have advanced the study of dentin proteins using the porcine animal model. The porcine animal model has the dual advantages of large developing teeth containing ample amounts of dentin proteins, and being available fresh and in quantity from meat processing facilities. Previously we cloned and characterized DSPP cDNA providing us with the deduced amino acid sequence of porcine DSPP excepting the redundant region in the DPP code, which was partially deleted during the cloning process (25). We determined that DSP is a proteoglycan that forms covalent dimers through an intermolecular disulfide bridge at Cys\(^{190}\) (21), and discovered and characterized dentin glycoprotein (DGP), a phosphorylated glycoprotein derived from the middle of DSPP between DSP and DPP (3). Our objectives in the present study were to map the cleavage sites in DSPP that are used to split DSPP into its component parts during odontogenesis, isolate dentin resident matrix metallocproteinases (MMPs), and use them to cleave high molecular weight DSPP-derived proteins. We assume that if a dentin-resident protease cleaves DSPP at specific sites in vitro, and those sites exactly match the ones used to generate DSPP-derived proteins in vivo, then that enzyme catalyzes those same DSPP cleavages in vivo.

Experimental Procedures

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Program at the University of Michigan.

**Generation of DGP anti-peptide antibodies** - A synthetic 14 amino acid DSPP peptide (Glu\(^{366}\) to Gln\(^{399}\); Cys-EAGKVEDRESKQ) from the DGP domain (Glu\(^{10}\) to Gln\(^{23}\)) was conjugated to the carrier protein KLH. This segment was selected, in part, because it is known to have no posttranslational modifications in the native protein. Antibodies were generated in rabbits using a protocol that included three immunizations, one test bleed, a fourth immunization, and a final bleed. Specific anti-peptide antibodies were purified from the final bleed using an affinity column containing the immobilized unconjugated DGP peptide, and ELISA tested before being used for Western blot analyses. The anti-peptide antibody was designated pDGP. We used the chicken polyclonal antibody raised against recombinant porcine DSP expressed in bacteria that extended from Ile\(^{1}\) to Lys\(^{366}\) (21), the rabbit antibody raised against the peptide Cys-QUEGVGEPGRGDP corresponding to the Gln\(^{175}\)-Asp\(^{186}\) segment of porcine DMP1 (26), and the chicken polyclonal antibody (ab14311, Abcam, Cambridge, UK) raised against a peptide extending from Pro\(^{60}\) to His\(^{188}\) of human MMP-2, which shares 96% identity with the pig homologue. The antibody dilution used in Western
blot analyses were: DSP, 1:50,000; DGP, 1:2000; DMP1, 1:10,000, and MMP-2, 1:1000).

Preparation of Dentin Powder - Tooth germs of permanent second molars were surgically extracted with a hammer and chisel from the maxillae and mandibles of six-month-old pigs, within minutes of each animal's termination at the Michigan State University Meat Laboratory (East Lansing, MI, USA). Typically two maxillary and two mandibular second molars were obtained from each animal. The developmental stage of the molars was advanced in crown formation, but prior to the onset of root formation. The soft tissue was removed with forceps and the enamel layer was scraped off with a curette. The remaining hard tissue was reduced to powder using a jaw crusher (Retsch, Newtown, PA, USA). About 32 molars (8 pigs) yielded 40 g of tooth powder, the amount used to extract dentin proteins.

Extraction of proteins from dentin powder - The 40 g of dentin powder was homogenized at 4 °C in 200 mL 50 mM Tris-HCl/4M guanidine buffer (pH 7.4) containing Protease Inhibitor Cocktail Set III (1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin and 10 μM pepstatin) (Calbiochem, San Diego, CA, USA) and 1 mM 1,10-phenanthroline (Sigma, St. Louis, MO, USA). Insoluble material was pelleted by centrifugation (15,900 x g) and homogenized twice more in guanidine buffer. The combined supernatants (soluble guanidine extracts) were dialyzed against water. About ~150 mg of protein precipitated during subsequent dialysis and was designated the guanidine-water pellet (GP). The GP fraction contained the bulk of the proteolytic activity. The guanidine-water supernatant (GS) after dialysis was lyophilized and contained ~65 mg of protein, mostly enamel protein cleavage products. The bulk of the tooth powder was not extracted by guanidine. This guanidine insoluble material was dialyzed against 4 L of 0.5 M acetic acid (HAc) containing 5 mM benzamidine (Sigma), 1 mM PMSF (Sigma) and 1 mM 1,10-phenanthroline (Sigma). Each day the calcium concentration in the reservoir was measured using the Calcium Reagent Set (Pointe Scientific, Canton, MI, USA) and the HAc was replaced. After ten days, the calcium ion concentration of the HAc reservoir fell below 0.2 mM, indicating that the tooth mineral was fully dissolved. The dialysis bag contents were centrifuged, and the supernatant designated the acid (A) extract. The A extract included DGP-containing cleavage products between 22- to 30-kDa from the middle region of DSPP. The pellet was extracted with 0.5 M acetic acid/2 M NaCl (AN), which dissolved dentin phosphoprotein (DPP) and DSP-containing proteins. The AN supernatant was fractioned to purify high molecular weight DSPP-derived proteins.

Purification of DSPP-derived proteins - Ninety mg of the AN extract or 25 mg of A extract was dissolved in 50 mM Tris-HCl/4M guanidine buffer (pH 7.4) and separated by size exclusion chromatography using a Sephacryl 200 column (1.6 cm x 100 cm, GE Healthcare, Chalfont St. Giles, UK) equilibrated with 50 mM Tris-HCl/4M guanidine buffer (pH 7.4) and run at a flow rate of 0.2 mL/min at 4 °C. Fractions were collected every 15 min, and absorbance at 280 nm determined. These procedures separated large DSPP-derived components (in the first two chromatographic peaks) from smaller contaminants in the AN extract and the smaller DGP-containing polypeptides from other constituents in the A extract.

Fractions corresponding to the first two chromatographic peaks (AN-S1 and AN-S2) were combined, dialyzed against water, lyophilized, resuspended in 0.05% trifluoroacetic acid (TFA) and fractionated by reversed phase-high performance liquid chromatography (RP-HPLC) using a Discovery (1.0 cm x 25 cm) C-18 column run at a flow rate of 1.0 mL/min and monitored at 230 nm (Buffer A: 0.05% TFA; Buffer B: 80% acetonitrile/buffer A). DSP components having successively higher molecular weight comprised peaks 3 through 5 (ANS-R3 to R5). Fraction 2 (ANS-R2) contained DPP, which was further purified by RP-HPLC using a POROS R2 (4.6 mm x 10 cm) column at a flow rate of 1 mL/min and monitored at 220 nm (Buffer A: 0.05% TFA; Buffer B: 80% acetonitrile/buffer A). DPP was found in the second of three POROS R2 column fractions (ANSR2-b).

Smaller DSPP-derived proteins including DGP-containing proteins were observed in the second size-exclusion fraction of the A extract (A-S2), which was dialyzed against water, lyophilized, resuspended in 0.05% trifluoroacetic acid (TFA) and fractionated by RP-HPLC using a POROS R2 (4.6 mm x 10 cm) column at a flow
rate of 1 mL/min and monitored at 220 nm (Buffer A: 0.05% TFA; Buffer B: 80% acetonitrile/ buffer A).

Release of N-linked Oligosaccharide Chains by Glycopeptidase A Digestion - The three purified DSP peaks (ANS-R3 to 5) (approximately 0.1 mg each) in 0.1 M citrate-phosphate buffer (pH 5.0) were incubated with 1 mU of glycopeptidase A (Seikagaku America, East Falmouth, MA, USA) containing the Protease Inhibitor Cocktail Set II (0.08 mM of AEBSF, 6.8 μM of Bestatin, 0.8 μM of E-64, 0.35 mM of EDTA and 8 μM of Pepstatin A; Calbiochem) at 37 °C for 48 h. At the end of the incubation period, three volumes of ice-cold ethanol were added to the reaction mixture, which was then centrifuged for 10 minutes at 10,000 x g. Both supernatant and pellet were lyophilized and stored at -80 °C. An aliquot (20 μg) of the pellet was electrophoresed on a 4-20% tris-glycine gel and analyzed by stains-all staining and Western blot analysis using the DSP antibody.

Purification of dentin matrix metalloproteinases - Because of the low abundance of proteases in dentin, we started the extraction with 150 g of dentin powder, which yielded ~1.1 g of guanidine-water pellet (GP). The GP fraction was fractionated by anion exchange chromatography using a Q-Sepharose Fast Flow column (1.6 cm x 20 cm, GE Healthcare) equilibrated with Buffer A: 50 mM Tris-HCl/6 M urea (pH 7.4). Proteins were eluted with buffer A for 20 h, a linear gradient of buffer B (A + 0.2M NaCl) for 5 h, and a linear gradient of buffer C (A + 2M NaCl) for 5 h at a flow rate of 0.2 mL/min at 4 °C, while monitoring the absorbance at 280 nm. MMP-2 and MMP-20 cosegregated in peak 4 (GPQ4-Q). The GP-Q4 fraction was further fractionated by size exclusion chromatography using a Sephacryl 200 column (1.6 cm x 100 cm, GE Healthcare) as described above. Both enzymes cosextracted to the first of five fractions (GPQ4-S1). Finally, this fraction was separated by RP-HPLC using a Discovery C18 column (10 mm ID x 25 cm, SUPELCO, Bellefonte, PA, USA) as described above. MMP-2 eluted in the fifth peak (GPQ4S1-R5) while MMP-20 eluted in the seventh (GPQ4S1-R7). The buffers of the MMP-2 and MMP-20 final fractions were replaced to 50 mM Tris-HCl buffer (pH 7.4) using a YM-3 membrane (Amicon, Beverly, MA, USA) and stored at -80°C for in vitro digestion of DSP-DGP and DPP. Recombinant hMMP-2 (Calbiochem) was used as a positive control in the identification of MMP-2 in Western blots.

In Vitro Digestion of DSP-DGP and DPP with MMP-2 and MMP-20 - Purified DSP fractions (ANS-R4 and -R5), DPP (ANSR2-b) and were directly dissolved with 0.5 ml of MMP-2 or MMP-20, CaCl₂ was added to achieve a final calcium concentration of 2 mM, and the digestion was incubated for 48 h at 37 °C. Aliquots (4 μg) taken at 0, 24 and 48 h were analyzed on 4-20% tris-glycine gels, and the degradation pattern was visualized stains-all staining and by Western Blot analysis using the DSP polyclonal antibody.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) - SDS-PAGE was performed using Novex 4-20% Tris-Glycine Gel (Invitrogen, Carlsbad, CA, USA). Samples were dissolved in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and electrophoresis was carried out using a current of 20 mAmp for 1.5 h. The gels were stained with Bio-Safe Coomassie Brilliant Blue (CBB; Bio-Rad) or stains-all (Sigma, St. Louis, MO, USA). The apparent molecular weights of protein bands were estimated by comparison with SeeBlue® Plus2 Pre-Stained Standard (Invitrogen).

Western immunoblots - Proteins were electro-transferred from SDS-PAGE onto a Hybond-P membrane (GE Healthcare), incubated with 5% blocking solution (BioRad) and primary antibody (hMMP-2, overnight; DSP, 2 h; DGP, 3 h; or DPI1 overnight) diluted as described above. The membrane was immunostained by chemiluminescent detection with ECL Plus Western Blotting Detection Kit (Amersham Pharmacia, Piscataway, NJ, USA).

Characterization of DSPP cleavage products proteins isolated from porcine dentin powder or from in vitro digestions - Porcine dentin powder fractions AS2-Rd, AS2-Re, AS2-Rf, and AS2-Rg containing the relatively low molecular weight (16- to 40-kDa) DSP or DGP positive proteins, and the in vitro digestions of the DSP-DGP fraction (ANS-R5) were resolved on 4-20% Tris-Glycine gels, electrotransferred to a Hybond- P membrane (GE Healthcare), lightly stained with CBB, and selected membrane strips containing specific DSP or DGP bands were excised and analyzed by N-terminal sequencing.
Amino Acid Analysis and Automated Edman Degradation - Purified DSP and DPP samples (0.02-0.03 mg) were hydrolyzed with 6 N HCl at 115 °C for 16 h. The amino acid analyses and Edman degradations were performed at the W.M. Keck Facility at Yale University using a Beckman Model 7300 ion-exchange instrument and Applied Biosystems Procise 494 cLC protein sequencer, respectively.

RESULTS

We started with developing second molars from the 6-month old pigs, which were nearing crown completion (Fig. 1A). By scraping the outside of the crown with a sharp curette, we removed all but the hardest enamel. The scraped crowns were pulverized to a powder (Fig. 1B) and sent through a series of three extractions: with guanidine, acetic acid, and acetic acid with salt (Fig. 1C). These extractions yielded four fractions, the guanidine-water pellet (GP), guanidine-water supernatant (GS), HAc extract (A), and the HAc/NaCl (AN) extract, which were analyzed by SDS-PAGE stained with CBB (Fig. 2A) and stains-all (Fig. 2B), by Western blotting using the DSP (Fig. 2C), DGP (Fig. 2D) and DMP1 (Fig. 2E) antibodies, and by gelatin (Figs. 2F and 2G) and casein zymography (Fig. 2H). These initial extractions separated many of the targeted dentin proteins from each other, and in ample quantities for further investigation. The proteases were collected in the GP fraction. Lower molecular weight (LMW) DSP- and DGP-positive cleavage products were in the A extract. Higher molecular weight DSP- and DGP-positive proteins were in the AN extract, as was DPP. Three dentin proteases were evident on zymograms, which corresponded to enzymes previously shown to be expressed by porcine odontoblasts: MMP-2 (27), MMP-20, (28), and KLK4 (29). We did not observe MMP-9, which corresponds to a 102-kDa gelatinase-positive band in rat dentin extracts (30). Instead, we observed a 98-kDa protease positive artifact band caused by DPP in the extract, which does not stain with CBB and therefore leaves an unstained band on the zymogram.

Purification of LMW-DSPP cleavage products in the A extract - Lower molecular weight DSP-positive proteins in the A extract (Fig. 2C) were fractionated by size exclusion chromatography (Fig. 3A). SDS-PAGE and Western blot analyses showed the target proteins remained grouped in the second fraction (Fig. 3B). The A-S2 fraction was separated by RP-HPLC, which produced a complex chromatogram yielding 14 different fractions (Fig. 3C). These fractions were analyzed by SDS-PAGE and Western blotting (Figs. 3D-3G). The DSP-positive proteins, which ranged from 22- to 38-kDa, grouped in fraction AS2-R2g (Fig. 3F). DGP-positive proteins, which ranged from 15- to 36-kDa, clustered in fractions AS2-R2d through AS2-R2f (Fig. 3G).

Identification of in vivo cleavage sites used to generate the of LMW-DSPP-derived proteins - The AS2-R2 fractions d through j were separated by SDS-PAGE, transblotted to a membrane, and selected bands (primarily DGP-positive bands) were excised and their N-terminal sequences determined (Fig. 4A). In addition, the AS2-R2g dentin fraction (containing LMW DSP-positive proteins) was rechromatographed, and separated into 5 fractions (Fig. 4B). Selected bands from these fractions (Fig. 4C) were analyzed by N-terminal sequencing (Fig. 4D). The resulting N-terminal sequences identified the sites at which the dentin proteins had been cleaved in vivo.

Three separate bands (marked 8, 10 and 11 in Fig. 4D), having apparent molecular weights between 25- and 35-kDa gave the DSP N-terminal sequence (1-IPVPQIKP). A 37-kDa DSP positive band (marked 12 on Fig. 4D) generated by cleavage near the middle of DSP gave the N-terminal sequence (200-xPPGxGEI). Six DSPP cleavage sites were identified within a 79 amino acid segment extending from Ser330 to Ser408. The N-termini of these DSPP cleavage products were: 330-SRSQRIED, 353-VTAISEPL, 360-LAIKSGQD, 362-IGKSDDKG, and 377-SGNRStIT and 408-SVKTQGEA. The apparent molecular weights of these six cleavage products descended from 25- to 10-kDa.

Isolation of DPP and high molecular weight (HMW) DSP - The largest DSPP-derived proteins in porcine dentin were in the AN extract and could be separated from smaller components in the AN extract by size exclusion chromatography (Fig. 5A). SDS-PAGE and Western blot analysis showed that DSP and DPP were together in the first two fractions (Fig. 5B;
AN-S1 and AN-S2), which were combined because of their similar profiles (data not shown) and separated into 5 fractions (Fig. 5C; ANS-R1 to ANS-R5) by C18 RP-HPLC. These fractions were analyzed by SDS-PAGE and Western blot analyses (Fig. 5D). ANS-R5 contained high molecular weight smears that were CBB, stains-all, DSP and DGP positive. These results suggested that DSP in fraction ANS-R5 is still attached to DGP (DSP-DGP). In contrast, fraction ANS-R4 contained a CBB, stains-all, and DSP-positive, smear that was DGP negative and had a somewhat lower average molecular weight than the contents of ANS-R5. This result suggested that the DSP in ANS-R4 had already been separated from DGP by proteolytic cleavages. ANS-R3 contained a CBB negative, stains-all positive smear of uncertain identity that did not react with our DSP, DGP and DMP1 antibodies. ANS-R2 contained the familiar CBB negative, stains-all positive DPP band at 98-kDa, along with a stains-all positive smear of likely DPP degradation products. This sample was characterized by Edman degradation with gave the known DPP N-terminal sequence: DDPNxxEESxG (x = blank cycle). To minimize the amount of DPP cleavage products in with the 98-kDa DPP, ANS-R2 was passed over a POROS R2 column and the final DPP fraction was designated ANS-R2b (Fig. 5E).

To gain more information about the contents of fractions containing high molecular weight DSPP-derived proteins, we determined their amino acid compositions and compared them to the compositions calculated from the porcine DSP and DSP-DGP deduced amino acid sequences (Table 1). Although the porcine DPP sequence is not known, the highly unusual richness of the ANS-R2 fractions in Asx and Ser are characteristic of DPP. The amino acid composition of the high molecular weight fraction positive for DSP and DGP (ANS-R5) was similar to that predicted for DSP-DGP, except that Asx and Ser were high. This suggests that either some of the DSP-DGP protein also contained all or part of the DPP domain, or a small amount of DPP degradation products were still present in the ANS-R5 fraction. Similarly, the amino acid composition of the high molecular weight fraction positive for DSP but negative for DGP (ANS-R4) corresponded to that predicted for DSP with the exceptions of Asx and Ser, which were high. As DSP can only be connected to DPP through DGP (which was absent), this finding suggests some DPP degradation products were still present in the ANS-R4 fraction. Curiously, ANS-R3 looked similar to DSP and DSP-DGP on the stains-all gel, only smaller, and gave an amino acid composition similar to DSP, but was negative for the DSP antibody and CBB staining. The molecular constituents of ANS-R3 are still unknown. We speculate this fraction contains a highly glycosylated DSP “core” that lacks the N- and C-terminal antigenic regions of DSP.

Isolation of MMP-2 and MMP-20 from porcine dentin - Three major proteases were evident in our extracts, MMP-2, MMP-20, and KLK4. These enzymes were all part of the guanidine extract that precipitated in the dialysis step (GP). To purify these proteases, we started with 150 g of dentin powder, or almost 4 times the amount shown in Fig. 1B. The GP extract was first fractionated by ion exchange chromatography (Fig. 6A). All of the dentin proteases eluted in the fourth peak (Q4), data not shown. The GP-Q4 fraction was separated into 5 fractions by size exclusion chromatography (Fig. 6B), which were analyzed by SDS-PAGE stained with CBB (Fig. 6C), and by gelatin (Fig. 6D) and casein zymography (Fig. 6E). MMP-2 and MMP-20 cosegregated in the first fraction (GPQ4-S1), while KLK4 was found in the second and third fractions (GPQ4-S2 and GPQ4-S3). To separate MMP-2 from MMP-20, GPQ4-S1 was divided into 7 fractions by RP-HPLC, and then analyzed by SDS-PAGE stained with CBB (Fig. 6F) and by gelatin (Fig. 6G) and casein (Fig. 6H) zymography. MMP-2 was detected in peak 5 (GPQ4S1-R5) by gelatin zymography; MMP-20 was found in peak 7 (GPQ4S1-R7) by casein zymography. We confirmed the gelatinase activity in GPQ4S1-R5 was MMP-2 by Western blot analysis using a commercially available MMP-2 antibody and recombinant hMMP-2 as a positive control (Fig. 6I). The gelatinase positive bands from porcine dentin on the zymograms comigrated with MMP-2 positive bands on the Western, and the top band comigrated with the recombinant hMMP-2 protein.

Digestion of DSP, DSP-DGP, and DPP with MMP-2 and MMP-20 from porcine dentin - ANS-R4 (DSP) and ANS-R5 (DSP-DGP) were digested with GPQ4S1-R5 (MMP-2) and
GPQ4S1-R7 (MMP-20) and the digestion products were characterized by SDS-PAGE stained with CBB and by Western blotting using the DSP antibody (Fig. 7A). Both MMP-2 and MMP-20 generated multiple lower molecular weight DSP-positive cleavage products, but only when digesting ANS-R5 (DSP-DGP), not ANS-R4 (DSP). The in vitro digests of DSP-DGP generated DSP-positive cleavage products that comigrated with the lower molecular weight DSP-positive cleavage products isolated from dentin powder (Fig. 7B and 7C). MMP-2 generated somewhat smaller DSP cleavage products than MMP-20. Neither MMP-2 nor MMP-20 cleaved ANS-R2-b (DPP) over a 48 h incubation (Fig. 7D).

Four of the lower molecular weight DSP cleavage products generated by MMP-2 and one by MMP-20 were characterized by N-terminal sequencing (Fig. 7E). Three of the MMP-2 cleavage products shared the same N-terminus (Ser^330), but varied in their apparent molecular weights (25-, 23- and 15-kDa), suggesting they differed in the position of their C-termini. The fourth was a 19-kDa product starting at I^362. MMP-20 cleaved DSP-DGP before Ser^277, generating dentin glycoprotein (DGP). All of the in vitro cleavage sites characterized by N-terminal sequencing were nearer to the C-terminal side of DSP-DGP, but not all of the cleavage sites generated by MMP-2 and MMP-20 generated CBB positive bands that could be cleanly excised and characterized by N-terminal sequencing.

**DISCUSSION**

In this study we undertook the largest scale isolation of dentin proteins yet reported. We developed an efficient system for the extraction of DSPP-derived proteins and have isolated hundreds of milligrams of DSP and DPP. Porcine DPP migrates as a dark purple, stains-all positive band at 98-kDa. Technically it is difficult to separate DPP from a smear of apparent DPP degradation products that also stain deep purple with stains-all. An unusual feature of DPP is that it does not stain with CBB. Because of this, DPP made a clear band on gelatin zymograms that we originally mistook for being MMP-9, a protease reportedly in rat dentin that migrates at a similar position (30). We did not detect MMP-9 in porcine dentin extracts. Other differences between rat and porcine dentin extracts were noted. Porcine DPP, like bovine and human DPP (31), appears as a single band on SDS-PAGE and does not display multiple highly, moderately, and low phosphorylated bands, as have been described in rat dentin (32,33). Finally, rat DSP comprises only 5-8% of dentin non-collagenous protein, while DPP is present in almost 10-fold greater abundance (50% of dentin non-collagenous protein) (34). In porcine dentin we find these two DSPP-derived proteins to be present in approximately equal amounts.

DSPP is synthesized as a chimeric protein, comprised of 3 parts: DSP-DGP-DPP. Despite using molars in an early stage of development, and diligently inhibiting proteolysis during the extractions, we could not isolate or even detect intact DSPP in our extracts. Without a DPP-specific antibody, however, it was not possible to rule out that small amounts of DSPP might be present. Full-length DPP isolated from porcine dentin powder was not antigenic in chicken (Lampire Biological Laboratories, Pipersville, PA, USA), and a porcine DPP synthetic peptide Cys-GNDSDSKEEAEEDN (Zymed Laboratories, Inc. San Francisco, CA, USA) was not antigenic in rabbit. We assume that the extensive posttranslational modifications of DPP interfere with its antigenicity. Our highest molecular weight DSPP-derived protein fraction was ANS-R5, which contained DSP-DGP, but little if any DSP-DGP-DPP. The amino acid composition of ANS-R5 was high in Asx and Ser, suggesting that some DSP-DGP protein might still be attached to DPP or part of DPP. On the other hand, Asx and Ser were also high in ANS-R4, which contains DSP without DGP and therefore this DSP cannot be linked to DPP. As ANS-R3 was also high in Asx and Ser, we suspect that diffuse DPP degradation products were present in all of the reversed phase fractions (ANS-R1 through ANS-R5) and that the high Asx and Ser values for ANS-R5 does not afford evidence that intact DSPP was present in that fraction. The analysis of DSPP-derived proteins in porcine dentin extracts indicates that DSP-DGP-DPP is first cleaved on the N-terminal side of Asp^108, splitting DSP-DGP from DPP. This cleavage is very rapid, so that virtually no intact DSPP is detected in the dentin extracts. Which protease catalyzes that cleavage is still undetermined, as we were unable to isolate intact DSPP protein to serve as a substrate to generate
this cleavage in vitro. What happens after the proteolytic release of DPP can be deduced from our characterization of DSP-DGP cleavage products in the dentin extracts.

Porcine DSP-DGP is a proteoglycan having 457 amino acids (Ile1 to Gly457). This protein is highly glycosylated, having potential N-linked glycosylations at positions 37, 67, 77, 136, 155, 161, 176, 315, and 382. However, only the glycosylations at Asn77, Asn155 and Asn382 have been confirmed at the protein level (35). DSP-DGP also has glycan attachments (chondroitin 6-sulfate) at Ser258 and/or Ser230 (21). These glycosylations are likely to protect regions of DSP-DGP that might otherwise be susceptible to proteolysis and precludes the use of mass spec analyses to deduce the C-terminals of the DSPP cleavage products. The positions of the DSP-DGP cleavage sites determined in this study with respect to potential glycosylation sites are summarized in Fig. 8.

Twelve different DSP-DGP cleavage products from developing porcine molars were characterized by N-terminal sequencing, which provided us with sufficient knowledge of the cleavage sites are used in vivo to determine if MMP-2 and MMP-20 play a role in the generation of DSPP-derived proteins in dentin. DSP-DGP was digested in vitro and four MMP-2 cleavage products and one MMP-20-generated cleavage product were characterized. MMP-20 performs the cleavage that separates DSP and DGP (before Ser77), and generates a series of 25- to 38-kDa DSP-positive cleavage products that closely correlate with in vivo products containing the DSPP amino-terminus (compare Fig. 7C lanes 1 and 3, and Fig. 4C bands 8, 10, and 11). These results demonstrate that MMP-20 processes DSP-DGP from both ends. MMP-2, on the other hand, cleaves DSP-DGP primarily within the C-terminal region of the DSP (at Ser130 and I162), releasing DGP-containing cleavage products we refer to as “extended DGPs”, and also made cuts within the DGP domain. The processing of DSP by MMP-2 and MMP-20 suggest these enzymes may serve overlapping functions. Molecular redundancy may explain the lack of conspicuous dentin phenotype in mice and men lacking MMP-20 (36-38).

DSP-DGP is cleaved in the dentin extracellular matrix by MMP-2 and MMP-20, generating DSP and DGP or extended DGP. These proteases continue to process and partially degrade the dentin proteins. DSP appears to be reduced to a highly glycosylated core, which is resistant but not immune to further cleavages. The generation of DSPP-derived proteins could serve both activation and degradation functions. The processing of DSP-DGP by MMP-20 and MMP-2 appears to us very similar to the processing of enamel proteins, such as amelogenin, by MMP-20 (39). MMP-20 processes amelogenins into a series of relatively stable cleavage products, but continues to degrade those cleavage products, allowing the enamel to achieve higher degrees of mineralization. Later in enamel formation, KLK4 is secreted as a way of rapidly degrading the protein MMP-20 leaves behind (40). We were not able to detect any proteolytic activity of MMP-20 or MMP-2 against DPP, and yet a smear of apparent DPP degradation products was evident in the matrix. Others have noted that DPP is degraded as dentin matures (41), and that DPP is virtually absent from mature human teeth (31). The mechanism of DPP degradation in vivo is completely unknown. Given the early stage of development of the porcine second molars used in this study, we were surprised by the amount of apparent degradation that DSPP had already sustained. Are the non-collagenous proteins in dentin degraded after serving their function, as in enamel, to create space for increased mineralization?

REFERENCES


**FOOTNOTES**

*We thank Mr. Tom Forton, Manager of the Michigan State University Meat Laboratory and members of the Michigan State University Department of Animal Science for their kind assistance in obtaining fresh developing molars from pigs processed at their facility. We thank Dr. Myron Crawford, director of W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University and Nancy Williams for the protein sequencing. We thank Zymed Laboratories, Inc. (San Francisco, CA, USA) for generation of the rabbit anti-peptide antibodies against porcine DGP. This investigation was supported by USPHS Research Grants DE12769, DE15846, and DE11301 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 29892.*

1The abbreviations used are: A ext; acetic acid extract; AN ext; acetic acid/NaCl extract; Ab; antibody; CBB; Coomassie Brilliant Blue; DD; dentin dysplasia; DGI; dentinogenesis imperfecta; DGP; dentin glycoprotein; DMP1; dentin matrix protein 1; DPP; dentin phosphoprotein; DSPP; dentin sialophosphoprotein; DSP; dentin sialoglycoprotein; GP ext; guanidine pellet extract; GS ext; guanidine supernatant extract; HAc; acetic acid; hMMP-2; human MMP-2; HPLC; high performance liquid chromatography; KLK4; kallikrein 4; LMW; lower molecular weight; MMP-2; matrix metalloproteinase 2 or gelatinase-A; MMP-20; matrix metalloproteinase 20 or enamelysin; Ppt; precipitate; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RP; reversed phase; Sup; supernatant; TFA; trifluoroacetic acid; U; units.

**FIGURE LEGENDS**

Fig. 1. Extracting matrix proteins from porcine dentin. A, surgically extracted second molars are in the crown formation stage and have a mesial-distal dimension of about 2 cm. B, 40 g of dentin powder in a 2-cup container obtained by pulverizing 32 second molars after scraping off the enamel. C, flow chart showing the procedures used to produce primary extracts for the purification of proteases and DSPP-derived proteins from dentin powder. Key: Sup, supernatant; Ppt, precipitate; GS, guanidine supernatant; GP, guanidine pellet; A, acid; AN, acid and salt.

Fig. 2. Primary dentin extracts. The four primary dentin extracts are the guanidine supernatant (GS), the guanidine pellet (GP), the acetic acid extract (A), and the acetic acid with NaCl (AN) extract. A and B: Porcine dentin powder extracts analyzed by SDS-PAGE stained with CBB and stains-all. Dentin phosphoprotein (DPP) is the CBB negative, stains-all positive band migrating at 98-kDa in the AN extract (a, arrowheads). C, D, and E: Western blots of SDS-PAGE using the DSP, DGP, and DMP1 antibodies. Lower molecular weight DSP cleavage products from 22 to 38-kDa are observed in the A extract (b, bracket). Higher molecular weight DSP- (c, bracket) and DGP-positive (d, bracket) proteins are observed in the AN extract. Most of the lower molecular weight DGP positive bands in A extract are also DSP positive, except the only DGP-positive proteins from 16 to 21-kDa (d, bracket). Dentin matrix
protein 1 (DMP1) was not detected in any of the dentin extracts. F, gelatin zymogram of dentin extracts. Strong gelatinase positive activities are evident at 60- to 65-kDa (MMP-2; f, arrowheads) and 30- to 36-kDa (KLK4; g, arrowheads) in the GP extract. A relatively weak band at 98-kDa was observed in the AN extract (a, arrowhead). G, gelatin zymograph of dentin extracts stained with stains all. Stains-all staining eliminated the weak, 98-kDa band (a, arrowhead) suggesting that this gelatinase-positive band was an artifact caused by the DPP band at 98-kDa, which does not stain with CBB. H, casein zymogram of dentin extracts, which shows a weak doublet at 41- and 44-kDa (MMP-20; h, arrowheads).

**Fig. 3.** Purification of LMW DSPP-derived proteins from the A extract. A, chromatogram of the acetic acid extract being separated into three fractions (S1 to S3) by size exclusion chromatography. B, Fractions A-S1 to A-S3 analyzed by SDS-PAGE stained with CBB (left) stains-all (middle) and by Western blotting (right) using the DSP antibody. All of the LMW DSP-positive proteins co-purified in the second (S2) fraction. C, Chromatogram of A-S2 being separated into 14 fractions (a through n) by C18 RP-HPLC. D to G: C18 fractions containing DSP-positive (fraction g) or DGP-positive (fractions d through g) analyzed by SDS-PAGE stained with CBB (D), stains all (E), and by Western blotting using the DSP (F) and DGP (G) antibodies.

**Fig. 4.** Isolation and N-terminal sequencing of LMW DSP and DGP bands. A, SDS-PAGE stained with CBB showing the contents of fractions AS2-Rd through AS2-Rj. Bands characterized by N-terminal sequencing (DGP-positive bands 1-4 and prominent stains-all positive band 13) are labeled. B, chromatogram of AS2-Rg, containing the LMW DSP positive bands, being separated into 5 fractions by a POROS RP-HPLC column. C, SDS-PAGE (left) and Western blot (DSP Ab, right) analyses of fractions AS2-Rg1 through AS2-Rg5. Two only DGP-positive bands (5 and 6), and six DSP-positive bands (7 to 12) were characterized by N-terminal sequencing. D, N-terminal sequences of the 13 dentin protein bands. Protein sequences 1 through 12 are all derived from DSPP. The prominent stains-all positive protein in AN2-Rj (band 13) corresponded to the N-terminus of porcine SPARC. The number at the start of each sequence corresponds to the number of the N-terminal amino acid in the deduced porcine DSPP or SPARC sequences for the secreted protein (excluding the signal peptide).

**Fig. 5.** Isolation of DSP, DSP-DGP, and DPP. A, chromatogram of the AN extract being separated into 4 fractions (ANS1 through ANS4) by a size exclusion column. B, Analysis of these fractions by SDS-PAGE stained with CBB (left), stains all (middle) and a Western blot using the DSP antibody (right). C, chromatogram of the combined ANS1 and ANS2 fractions being separated into 5 parts (ANS-R1 through ANS-R5) by RP-HPLC. D, SDS-PAGE stained with CBB and stains all, and Western blots immunostained with the DSP, DGP, and DMP1 antibodies. E, chromatogram of ANS-R2, containing DPP and a smear of likely DPP degradation products, being separated into three fractions (ANS-R2a through ANS-R2c) using a POROS RP-HPLC column. F, SDS-PAGE stained with CBB (left) and stains all (right). The final purified DPP was fraction ANS-R2b.

**Fig. 6.** Isolation of MMP-2 and MMP-20. A, chromatogram of the GP extract being separated into 5 fractions (GP-Q1 through GP-Q5) by ion exchange chromatography. B, chromatogram of GP-Q4, containing gelatinase-A (MMP-2), enamelysin (MMP-20), and kallikrein 4 (KLK4), as it is separated into 5 fractions (GPQ4-S1 through GPQ4-S5) by size exclusion chromatography. C, SDS-PAGE of GPQ4-S1 through GPQ4-S5 stained with CBB. D, gelatin zymogram of the size exclusion fractions showing MMP-2 to be in fraction 1 (GPQ4-S1) and KLK4 in fractions 2 (GPQ4-S2) and 3 (GPQ4-S3). E, casein zymogram showing MMP-20 in fraction 1 (GPQ4-S1). F, SDS-PAGE stained with CBB of 7 fractions (GPQ4S1-R1 through GPQ4S1-R7) obtained by the RP-HPLC separation of GPQ4-S1. G, gelatin zymogram of GPQ4S1-R1 through GPQ4S1-R7 showing MMP-2 to be in fraction 5 (GPQ4S1-R5). H, casein zymogram showing MMP-20 in fraction 7 (GPQ4-S7). I, Analyses of fraction GPQ4S1-R5 by SDS-PAGE stained with CBB, gelatin zymography, and Western blotting using a commercial MMP-2
antibody. Lane +C is the positive control on the Western blot showing recombinant hMMP-2 comigrates with the top band of porcine MMP-2 and immunoreacts with the antibody.

**Fig. 7.** Cleavage of DSP and DSP-DGP in vitro. A, SDS-PAGE stained with CBB (left) and Western blot immunostained with the DSP antibody (right). The top panel shows a time course for the digestion of DSP (ANS-R4) and DSP-DGP (ANS-R5) by MMP-2; the bottom panel shows the same proteins digested by MMP-20. The three time points shown are at 0, 24, and 48 h, as well as a 48 h control (C) with no protease added. Lower molecular weight DSP-positive cleavage products are only evident in the DSP-DGP digestion. B, SDS-PAGE and C, Western blot using the DSP antibody comparing the lower molecular weight DSP-positive proteins extracted from developing pig molars (AS2-R2g, lanes 1) to the MMP-2 digestion of DSP-DGP (ANS-R5, lanes 2), and the MMP-20 digestion of DSP-DGP (ANS-R5, lanes 3). Note the correspondence between the lower molecular weight DSP cleavage products in fraction AS2-R2g and those generated by MMP-2 and MMP-20. D, SDS-PAGE stained with stains-all showing a time course for the digestion of DPP (ANSR2-b) by MMP-2 (left) and MMP-20 (right). As for the DSP digestions, the time points are at 0, 24, and 48 h, and include 48 h control (C) with no protease added. Neither enzyme generated a perceptible cleavage product, even after 48 h. E, list of the five N-terminal sequences determined for the CBB positive digestion products (a through e) in B (arrowheads).

**Fig. 8.** Proteolysis of DSP-DGP in vivo and in vitro. A, bar representing primary structure of porcine DSP-DGP. The numbers above the bar numbers mark potential N-linked glycosylation sites (Asn$^{37}$, Asn$^{67}$, Asn$^{77}$, Asn$^{136}$, Asn$^{176}$, Asn$^{315}$, and Asn$^{382}$) and glycan attachment sites (Ser$^{285}$, and Ser$^{250}$). Confirmed modification sites are in bold. Below the bar are cleavage sites discovered by characterizing DSPDPP-derived cleavage products from developing porcine molars. Arrows indicate the approximate locations of deduced cleavage sites inferred from the molecular weights of cleavage products having defined N-termini. Solid bars show the positions of the DSP and DGP antigens used to raised the antibodies used in this study. B, Bars showing the 12 DSPP cleavage products isolated from in vivo and characterized by N-terminal sequencing (Fig. 4). These bars align with the DSP-DGP structure in $A$. Exact matches between in vivo and in vitro cleavage products are indicated (i.e., in vivo band 1 from Fig. 4 exactly matches MMP-20 band “e” from Fig. 7). Note that in vivo cleavage products 1 through 4 do not extend far enough on their N-termini to be recognized by the DSP antibody. The only in vitro cleavage product that was not identical to a characterized in vivo cleavage product was MMP-2 band d (hollow bar). This band matched several DSP cleavage products (6, 7 & 9) on its N-terminus (Ser$^{330}$). We suspect MMP-2 band d extended to Gly$^{307}$, which is the cleavage site that generated band 2. This band is DGP-positive, so its C-terminus must extend to contain the DGP epitope, but cannot extend much further due to its small size (15-kDa).
Table 1. Amino acid compositions of ANSR2-abc through ANS-R5. The purest 98-kDa DPP fraction is R2b. DSP without DGP is in fraction R4. DSP-DGP is in fraction R5. The deduced amino acid compositions of porcine DSP and DSP-DGP are provided on the right.

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Figure 1

Dentin Powder

50 mM Tris/4 M Guanidine (pH 7.4)

Sup Ppt

(GP ext)

Sup Ppt

(GS ext)

Dialysis (water)

Sup Ppt

0.5 M acetic acid demineralization

0.5 M acetic acid

(A ext)

Sup (AN ext) Ppt

0.5 M acetic acid/2 M NaCl

Sup (AN ext) Ppt

A

B

C

Dentin Powder

Sup Ppt

50 mM Tris/4 M Guanidine (pH 7.4)

Sup (GS ext) Ppt (GP ext)

Sup Ppt

Dialysis (water)

Sup Ppt

0.5 M acetic acid demineralization

Sup (A ext) Ppt

0.5 M acetic acid

Sup (AN ext) Ppt

0.5 M acetic acid/2 M NaCl

Sup (AN ext) Ppt
Figure 2

Panel A shows a gel with bands labeled with letters a, c, and d. Panel B displays a gel stained with CBB only, with bands labeled with letters a, c, b, and d. Panel C contains a gel stained with CBB + stains-all, with bands labeled with letters a, c, b, e, and f. Panel D presents a gel stained with CBB only, with bands labeled with letters a, c, b, e, and f. Panel E shows a gel stained with CBB only, with bands labeled with letters a, c, b, e, and f.
Figure 3

A

B

C

D

E

F

G

S1 S2 S3 S1 S2 S3 S1 S2 S3

250 148 98 64 50 36 22 16 6

DSP-Ab

148 250 98 64 50 36 22 16 6
Figure 4

D

1: DSPP 377-SGNRSxIT
2: DSPP 408-SVKTQGEA
3: DSPP 362-IGKSQDKG
4: DSPP 360-LAIGKSQD
5: DSPP 353-VTAISEPL
6: DSPP 330-SRSQRIED
7: DSPP 330-SRSQRIED
8: DSPP 1-IPVPQIKP
9: DSPP 330-SRSQRIED
10: DSPP 1-IPVPQIKP
11: DSPP 1-IPVPQIKP
12: DSPP 200-xPPGxGEI
13: SPARC 1-APQQEALP
Figure 5
A

DSP Antigen $I^1$ to $K^{368}$

MMP-20 (band e) 1
MMP-2 (band c) 3
MMP-20 (25 to 38-kDa DSP-positive bands)
MMP-2 (band d) 5
MMP-2 (band b) 6 & 9
MMP-2 (band a) 7

B

DGP Antigen $E^{386}$ to $Q^{199}$

8
10
11

(25 to 38-kDa DSP-positive bands)
Dentin sialophosphoprotein is processed by MMP-2 and MMP-20 in vitro and in vivo
Yasuo Yamakoshi, Jan C-C. Hu, Takanori Iwata, Kazuyuki Kobayashi, Makoto Fukae and
James P. Simmer

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