Characterization of the Interaction Between Bone Sialoprotein and Type I Collagen

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Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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CHARACTERIZATION OF THE INTERACTION BETWEEN BONE SIALOPROTEIN AND TYPE I COLLAGEN

(Thesis format: Integrated Article)

by

Rose Yee

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

The mechanism of biomineralization is unknown. In bone, it has been proposed that an acidic phosphoprotein of the extracellular matrix (ECM) is important in the nucleation of hydroxyapatite (HA). The mineralized tissue protein, bone sialoprotein (BSP), has been shown to be a potent nucleator of HA and this activity is increased upon binding to type I collagen. The collagen-binding domain of BSP has been determined to be a highly conserved region (spanning amino acid residues 18-45); however, the area of collagen involved in this interaction is unknown.

In this study, a chemical cross-linking method was initially utilized to map the BSP-binding region on collagen. These experiments involved the incubation and interaction of five different single-cysteine recombinant BSP (1-100) mutants and type I collagen in the presence of two sulphydryl-reactive, photoactivatable cross-linking reagents: ρ-azidophenacyl bromide (APB) and N-[4-(ρ-azidosalicylamido) butyl]-3’-(2’-pyridyldithio) propionamide (APDP). However, due to the low yield observed from SDS-PAGE in all instances, the complexes were not further characterized.

As an alternate approach, rotary-shadowing transmission electron microscopy (TEM) was used. This involved incubation of recombinant BSP (rBSP) with type I collagen, rotary-shadowing with platinum and analysis by TEM. Rotary-shadowing of BSP revealed a 10-nm globular structure that is linked to a thread-like structure of ~25 nm while type I collagen appeared as semi-flexible, rod-like structures. Combining BSP and collagen showed a locus of interaction at a site that averages approximately 31% from the N-terminus, based on the site of interaction of an antibody to the N-terminus of the collagen molecule. Due to the observed variability, BSP interacts with collagen between residues 255-375. Based on the locus of interaction, the arrangement of the collagen fibril, the highly flexible structure of
BSP and the hydrophobic nature of the BSP-collagen binding interaction, we postulate that the N-terminal hydrophobic sequence (residues 18-45) of BSP binds collagen in an overlap region at a site that is rich in hydrophobic residues, while the two glutamic acid-rich regions extend into the gap region of the adjacent collagen molecule.

Within the collagen-binding domain of BSP, there is a highly conserved thrombin-cleavage site at Arg27-Tyr28. Thrombin cleaves rat BSP in the middle of the collagen-binding domain (residues 18-45), which effectively abolishes collagen-binding, implying physiological significance. To address the relevance, incubation of thrombin with BSP and fibrillar collagen and with demineralized bone chips was performed to determine if BSP was released from its complexes. Analysis with SDS-PAGE and western blotting demonstrated that rBSP bound to type I collagen fibrils is susceptible to thrombin cleavage. Similarly, endogenous BSP present in demineralized bone chips is also released from these chips with thrombin incubation. Since BSP appears to enhance osteoprogenitor cell migration and differentiation, this suggests that, upon injury, the release of BSP from bone may provide signals to initiate the repair process. In summary, the results from these studies are important in better understanding the BSP-collagen interaction and its role in bone formation and mineralization.

**Keywords**  bone sialoprotein, phosphoprotein, SIBLING proteins, type I collagen, biomineralization, hydroxyapatite nucleation, chemical cross-linking, electron microscopy, protein-collagen interaction, thrombin
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<td>Definition</td>
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<tr>
<td>ADAM</td>
<td>adamalysin-related membrane proteases</td>
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<tr>
<td>AEBSF</td>
<td>4-(2-Aminooethyl) benzencesulfonfyl fluoride hydrochloride</td>
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<td>AP-1</td>
<td>activator protein-1</td>
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<td>APB</td>
<td>ρ-azidophenacyl bromide</td>
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<td>APDP</td>
<td>N-{4-(ρ-azidosalicylamido)butyl]-3’-(2’-pyridyldithio) propionamide</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>BMF</td>
<td>bone mineralization foci</td>
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<td>BMPs</td>
<td>bone morphogenetic proteins</td>
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<td>BSP</td>
<td>bone sialoprotein</td>
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<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
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<td>DMP-1</td>
<td>dentin matrix protein-1</td>
</tr>
<tr>
<td>DPP</td>
<td>dentin phosphophoryn</td>
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<td>DSPP</td>
<td>dentin sialophosphoprotein</td>
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<tr>
<td>DSP</td>
<td>dentin sialoprotein</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<td>gCK</td>
<td>golgi casein kinase</td>
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<tr>
<td>GRGDS</td>
<td>Gly-Arg-Gly-Asp-Ser</td>
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<tr>
<td>GuHCl</td>
<td>guanidine hydrochloride</td>
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<tr>
<td>HA</td>
<td>hydroxyapatite</td>
</tr>
<tr>
<td>HPR</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption ionization time-of-flight</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEPE</td>
<td>matrix extracellular phosphoglycoprotein</td>
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<tr>
<td>MM</td>
<td>molecular modeling</td>
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<tr>
<td>MMP</td>
<td>metalloprotease</td>
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<td>MSCs</td>
<td>mesenchymal stem cells</td>
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<td>m/z</td>
<td>mass/charge</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>nBSP</td>
<td>native (bone-derived) bone sialoprotein</td>
</tr>
<tr>
<td>NCPs</td>
<td>non-collagenous proteins</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>rBSP</td>
<td>recombinant bone sialoprotein</td>
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<tr>
<td>OI</td>
<td>osteogenesis imperfecta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>OPN</td>
<td>osteopontin</td>
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<tr>
<td>PAR-1</td>
<td>protease-activated receptor 1</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PEDF</td>
<td>pigment epithelium-derived factor</td>
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<tr>
<td>PG</td>
<td>proteoglycan</td>
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<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<td>phosphotungstic acid</td>
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<td>post-translational modifications</td>
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<td>polyvinylidene fluoride</td>
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<td>rBSP (1-100)</td>
<td>peptide of bone sialoprotein containing the first 100 amino acids</td>
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<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
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<td>sulfo-SBED</td>
<td>sulfosuccinimidyl-2-[6-(biotinamido)-2- (p-azidobenzamido)-hexanamido] ethyl-1,3-dithiopropionate</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium-dodecyl–sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SIBLING</td>
<td>small integrin binding ligand N-linked glycoproteins</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscop</td>
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<tr>
<td>TEMED</td>
<td>$N, N', N''$-tetramethylethane-1, 2-diamine</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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1.1 GENERAL INTRODUCTION

Bone is a specialized connective tissue consisting of three main components: mineral, cells and extracellular matrix (ECM). Bone undergoes constant change through remodeling, a process involving the resorption of bone and its reformation. Bone has three primary functions: (1) mechanical support, (2) protection of vital organs, and (3) storage of calcium and phosphate ions as a reserve which are essential for homeostasis (1, 2). Within bone, there are three phases: a cellular, organic and inorganic phase.

The cellular phase consists of three different bone cells: osteoblasts, osteoclasts and osteocytes (3). The organic phase comprises predominantly type I collagen (90%) and non-collagenous proteins (NCPs) such as osteopontin (OPN) and bone sialoprotein (BSP). The inorganic phase comprises 65% of the total bone weight and is composed primarily of hydroxyapatite crystals [HA; Ca_{10}(PO_{4})_{6}(OH)_{2}] which contribute to the mechanical rigidity of bone.

Biomineralization is a complex, yet poorly characterized process that involves many factors. Precise mechanisms of initiation, growth and packing of crystals are unknown. Several mechanisms for biomineralization have been postulated, including the proposal that an acidic phosphoprotein present in the ECM of mineralized tissues, when bound to type I collagen, will induce the nucleation of HA (4). BSP is an acidic non-collagenous phosphoprotein that is expressed primarily in mineralized tissues (5) and has been shown to be a potent nucleator of HA (6, 7). It is postulated that BSP is secreted into the ECM by osteoblasts, binds to type I collagen in the gap regions or the regions adjacent to it, and mediates mineral formation (Figure 1.1).
Figure 1.1 Model of BSP-mediated hydroxyapatite nucleation.

(A) The hydroxyapatite crystals (HA) are deposited onto a type I collagen-rich matrix. The collagen matrix neither promotes nor inhibits HA nucleation, but rather acts as the scaffold upon which HA crystals are deposited. BSP is a potent nucleator of HA with the two glutamic acid-rich regions (brown) being responsible for the activity and this nucleation activity is increased 10-fold when bound to type I collagen. Furthermore, BSP is postulated to bind to type I collagen (purple) in the gap regions or regions adjacent to it where early mineral formation is seen to occur. (B) Electron micrograph of calcified turkey tendon showing initial mineral formation occurring in the gap regions of collagen. [Adapted from (8)]
1.2 BONE DEVELOPMENT

The bones of the modern human skeleton are formed by two different processes: endochondral ossification and intramembranous ossification (9, 10). Long bones of the body are formed through endochondral ossification while the flat bones form through intramembranous ossification. Both processes involve condensation of mesenchymal stem cells (MSCs), differentiation of these cells, secretion of an osteoid (uncalcified bone matrix) and the initial formation of woven bone that is eventually remodeled to mature lamellar bone. The main difference between the two bone development processes is the presence of a cartilaginous intermediate in endochondral ossification (9).

1.2.1 Skeletal Cell Types

1.2.1.1 Osteoblasts

Osteoblasts are derived from MSCs, more specifically, the osteoprogenitor cells within the periosteum and bone marrow. Differentiation into osteoblasts is induced by various growth factors such as bone morphogenetic proteins (BMPs) and once differentiated; these cells display a range of genetic markers including Osterix, alkaline phosphatase and BSP. Osteoblasts are responsible for synthesizing the osteoid matrix via the secretion of collagenous and non-collagenous proteins (10).

1.2.1.2 Osteocytes

Osteocytes are the most abundant cells in bone tissue, comprising over 90% (11, 12). These cells are derived from mature osteoblasts that become engulfed in the mineralized tissue. Osteocytes are interconnected by long cytoplasmic extensions known as canaliculi and reside in spaces called lacunae (11, 13). These canaliculi function as routes for nutrients and oxygen transport and exchange. Additionally, osteocytes are
thought to act as mechanosensors such as for strain/stress and detecting fractures within the bone, and are crucial to the bone-remodeling process.

1.2.1.2 Osteoclasts

Osteoclast precursors are haematopoietic in origin, arising from the spleen and bone marrow. Osteoclast precursors are derived from the monocyte/macrophage lineage. Osteoclasts are multinuclear (containing 10-20 nuclei with 1-2 nucleoli in each nucleus), and are responsible for bone remodeling in the process known as bone resorption (12).

1.2.2 Intramembranous Ossification

Intramembranous ossification is the process for formation of flat bones, which include the skull and mandible. A cartilaginous intermediate is not present in this process. MSCs condense within a highly vascularized area of embryonic tissue and differentiate directly into pre-osteoblasts and eventually osteoblasts in regions known as ossification centres. Osteoblasts begin to secrete an osteoid matrix rich in type I collagen. Calcification traps many of the osteoblasts in the mineralized tissue where they differentiate into osteocytes. Osteoblasts and osteocytes deposit non-collagenous proteins and type I collagen aggregate randomly to form woven bone. Most of the woven bone is eventually remodeled and replaced by mature lamellar bone by clustering type I collagen fibrils in parallel arrays (9).

1.2.3 Endochondral Ossification

Endochondral ossification is a multi-step process requiring the formation and degradation of a cartilaginous intermediate that serves as template for the formation of long bones in the human body (9). MSCs condense, proliferate and differentiate into chondrocytes all while depositing a cartilaginous ECM comprising of mainly type II
collagen and cartilage-specific matrix proteins and proteoglycans. The tissue grows axially following the growth plates present at each end of the forming tissue. This process continues until the growth plates close at puberty.

In the process of mineralization, chondrocytes mature into hypertrophic chondrocytes and undergo apoptosis. The hypertrophic chondrocytes express proteins distinctly different from those expressed by chondrocytes, including collagen type X, BSP, matrix Gla protein and OPN (14). Following this event, vascular invasion allows the recruitment of osteoclast precursors from the circulation which then proceed to resorb the calcified cartilaginous matrix and induce the remodeling of the tissue mediated by osteoblasts. Osteoblasts synthesize osteoid via secretion of both collagenous and non-collagenous proteins. Following calcification, woven bone is initially formed and is eventually remodeled and replaced with mature lamellar bone.

1.2.4 Bone Repair

Bone repair occurs through a sequence of four stages: inflammation, soft callus formation, hard callus formation and remodeling. In response to bone injury, the blood coagulation cascade is activated to form a haematoma. Over two to three days, the haematoma is gradually replaced by granulation tissue. Progenitor cells of the periosteum (membrane of connective tissue that lies the outer surface of bone) nearest the fracture site differentiate into chondrocytes and synthesize cartilage, while progenitor cells further away differentiate into osteoblasts and synthesize osteoid. Together, these processes form the soft callus. Following calcification of the osteoid, a hard callus of woven bone is formed, while the cartilage becomes hyaline cartilage. Eventually, a union of the fracture gap occurs and the woven bone is remodeled by osteoclasts and osteoblasts to eventually
form mature lamellar bone (15). Bone remodeling will continue until the form and strength of the bone are restored to conditions prior to the fractured state.

If the injury is too severe that natural healing cannot repair the bone fracture, then surgical procedures may help in the healing process. For example, metal scaffolds (primarily titanium) are often used to hold the fractured bones in place to facilitate the bone healing. Additionally, the metal scaffolds may be coated with components that have been shown to increase bone repair such as HA, type I collagen, BSP or BMP (16).

1.3 BIOMINERALIZATION

Biomineralization refers to the process by which organisms use dissolved ions to form mineral crystals (17). Biological organisms have been using minerals for over 3.5 billion years and the earliest evidence of this process is the fossil of stromatolites entrapped in sedimentary rocks. Mineralized skeletal structures first appeared approximately 540 million years ago (18) and are either comprised of calcium carbonate or calcium phosphate. Calcium carbonate-based skeletal systems are found primarily in invertebrates such as molluscs and crustacean, whereas a crystalline form of calcium phosphate in the form of HA, is found in bones and teeth.

By transmission electron microscopy (TEM), HA crystals found in bone, dentin and mineralized tendon tend to be plate-like, with average dimensions of ~45 nm in length, ~25 nm in width and 3-6 nm thick (3, 19). Moreover, the c axes of the HA crystals are aligned with the collagen fibril axis (20).

Today, biomineralization occurs in all five biological kingdoms resulting in diverse array of minerals for skeletal, taxis and protective functions (17). Biomineralization still remains an active area of research as precise mechanisms of
initiation, growth and packing of crystals are unknown. However, it is believed that this process is tightly regulated by secreted proteins in the human body, as spontaneous calcifications are rare in healthy individuals. While there are several postulated mechanisms for biomineralization, including the involvement of matrix vesicles (21, 22) and inhibition mechanisms involving the removal of mineralization inhibitors (23-26), one mechanism to account for bone mineralization that has significant support in the literature is discussed below.

1.3.1 Organic Matrix-Mediated Mineralization

Organic matrix-mediated biomineralization involves the presence of a surface that promotes nucleation of crystals (27). The surface chemistry of the organic matrix is imperative to crystal formation and growth. In regards to the proteins found in the organic matrix, the surface chemistry is dictated by the primary and secondary structure and/or post-translational modifications (PTMs) (28). To date, proteins identified to be mediators of crystal formation and growth are highly acidic and phosphorylated (29).

In organic matrix-mediated biomineralization, there are four steps involved in crystal formation. The first two steps involve the sequestration and adsorption of $\text{Ca}^{2+}$ and $\text{PO}_4^{3-}$ ions onto an organic matrix leading to the nucleation and formation of nanometer-sized particles and eventually critical nuclei. The two following steps involve the stabilization of the critical nuclei and as a result, the formation of HA crystals (27).

In bone, it is proposed by some that mineralization occurs through a process known as epitactic nucleation where a protein mimics a crystal face resulting in the formation of a critical nucleus (17). More specifically, the proposed mechanism involves type I collagen acting as the pre-existing surface upon which the ions are deposited and
nucleated by acidic proteins (4, 30-32). BSP is an acidic non-collagenous phosphoprotein that is expressed primarily in mineralized tissues (5) and has been shown to be a potent nucleator of HA (6, 7, 33). BSP will be discussed in greater detail later in this chapter.

1.4 ORGANIC PHASE

The organic phase of bone comprises 30% of the total bone weight and is deposited primarily by osteoblasts and to a lesser extent, osteocytes. It is composed predominantly of type I collagen (90%) and NCPs such as OPN and BSP. Type I collagen neither promotes nor inhibits HA nucleation, but merely acts as a structural scaffold upon which HA are deposited. However the NCPs, specifically the SIBLING proteins (discussed later in this section), are proposed to be important in bone mineralization.

1.4.1 Type I Collagen

There are more than 29 types of collagen, with type I collagen being the most abundant in the human body. Dividing into subgroups based on the polymeric structures assembled, type I collagen belongs to the fibril-forming collagen superfamily, which includes type II, III, V and XI collagen. Type I collagen is a cationic molecule with an isoelectric point of 8.3. Type I collagen is found in bone, dentin, skin, tendon, ligaments and cornea and its expression is primarily restricted to cells that form these tissues including osteoblasts and fibroblasts (34).

1.4.1.1 Type I Collagen Structure

Type I collagen is comprised of three left-handed polypeptides – two identical $\alpha_1$ chains and one $\alpha_2$ chain. Each polypeptide comprises approximately 1,000 amino acid residues and is approximately 100 kDa (34, 36, 37). The three polypeptides wind together
to form a right-handed triple-helical structure with short non-helical telopeptides at both ends (Figure 1.2A) (37, 38). Therefore, the molecular weight of the full, triple-helical collagen structure is approximately 300 kDa.

Type I collagen has a characteristic repeating Gly-X-Y sequence, where X is commonly proline and Y is commonly 4-hydroxyproline. These residues are important in limiting the rotation of the polypeptide chains (34, 35). The 4-hydroxyproline residue is also involved in the stabilization of the triple-helix by forming hydrogen bonds and water bridges (34). The glycine residue at every third amino acid along each chain is crucial for the formation of the helix turn, since glycine has the smallest side chain and can fit within the centre of the triple helix, whereas the side chains of all the other amino acids cannot without disrupting the triple helix (35).

**1.4.1.2 Type I Collagen Fibril Formation**

Type I collagen monomers, which are approximately 300 nm in length and 1.4 nm in diameter, spontaneously aggregate forming fibrils resulting in overlap and gap regions with a periodicity (D) of 67 nm or 234 residues (Figures 1.2A and 1.2B) (37, 39). This arrangement is referred to as a staggered array or the Hodge and Petruska model (40). Each overlap region is approximately 27 nm (0.4 D) while each gap region is approximately 40 nm (0.6 D). In addition, the pore space between adjacent type I collagen molecules is 0.24 nm. The characteristic banding pattern resulting from the overlap and gap regions can be observed by negative staining of the collagen fibrils (Figure 1.2C) (37, 39). Moreover, positive staining of type I collagen fibrils with phosphotungstic acid (PTA) results in twelve bands within a D-period that are designated as a<sub>1</sub>-a<sub>4</sub>, b<sub>1</sub>, b<sub>2</sub>, c<sub>1</sub>-c<sub>3</sub>, d, and e<sub>1</sub> and e<sub>2</sub> (Figures 1.2D and 1.2E). These bands are a result of
the uptake of PTA anions by positively charged amino acids (i.e. lysine, hydroxylysine, arginine, and to a lesser extent, histidine) (41, 42). The gap region of the collagen fibril consists of $a_{3,2,1}, e_{2,1}, d$ and $c_3$ while the overlap region consists of $c_{2,1}, b_{2,1}$, and $a_{4,3}$.

Side-to-side intermolecular interactions, rather than end-to-end interactions, are important in maintaining the integrity of the collagen fibril (39). In addition, the non-helical telopeptides are important for stabilizing the collagen fibrils by facilitating intermolecular cross-links (39). Interestingly, early mineral formation has been observed in the gap regions of collagen fibrils.

There are many proposed models of the 3-D organization of type I collagen, however; there is one model that has significant support in the literature. Landis et al., proposed that the packing of consecutive 2-D collagen arrays into 3-D assemblages results in the formation of channels or gaps. These channels or gaps (with pore sizes of 0.24 nm) are postulated to be the sites of nucleation (43).

Although the structure of type I collagen is quite complex, the structure is physiologically relevant. To date, approximately 200 mutations have been characterized in the two genes, COL1A1, COL1A2, encoding the 2 different $\alpha$ chains of type I collagen. These mutations result in severe pathological conditions such as osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (44).
Figure 1.2 Type I collagen structure.

(A) Triple-helical type I collagen monomers spontaneously aggregate forming fibrils which result in (B) overlap and gap regions with a D-periodicity of 67 nm. (C) The characteristic banding pattern resulting from the overlap and gap regions are observed by negative staining with sodium phosphotungstic acid (1 %, pH 7). (D) The collagen fibrils were positively stained with phosphotungstic acid (1 %, pH 3.4) and then uranyl acetate (1 %, pH 4.2) resulting in (E) twelve staining bands. [Adapted from (45)]
1.4.1.3 Biosynthesis of Type I Collagen

The fibril-forming collagen family is first synthesized in the endoplasmic reticulum (ER) as soluble procollagen. They are then converted into collagen molecules via a series of enzymatic reactions. Proline residues at the Y position of the Gly-X-Y repeat and lysine residues undergo hydroxylation to 4-hydroxyproline and hydroxylysine by prolyl-4-hydroxylase and lysyl hydroxylase (46). Some of the proline residues at the X position are converted to 3-hydroxyproline by prolyl-3-hydroxylase. In terms of the hydroxylysine residues, galactose or both galactose and glucose are added to selected residues. Once the C-terminal propeptides associate and form intrachain and interchain disulfide bonds, the triple-helical conformation starts to assemble and propagate toward the N-terminus in a zipper-like manner.

Following or during secretion into the ECM, cleavage of N- and C- propeptides by procollagen N- and C- proteinases allows for the self-assembly of collagen monomers into collagen fibrils (37, 47). Furthermore, to stabilize the collagen fibrils, the enzyme lysyl oxidase converts selected lysine and hydroxylysine residues (found in the non-helical telopeptides) to aldehyde derivatives leading to cross-link formation.

1.4.2 The SIBLING Protein Family

The ECM contains many non-collagenous proteins including the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) protein family (48, 49). Members of the SIBLING protein family include: OPN, dentin matrix protein 1 (DMP-1), dentin sialophosphoprotein [DSPP – under physiological conditions, DSPP is cleaved by PHEX forming dentin sialoprotein (DSP) and dentin phosphophoryn (DPP)], matrix extracellular phosphoglycoprotein (MEPE) and bone sialoprotein (BSP) (48, 50).
The SIBLING proteins are expressed predominantly in mineralized tissues and are proposed to have important roles in mineralization. In bone and dentin, BSP and OPN exist as full-length forms, whereas DMP-1 and DSPP exist as distinct proteolytic-derived large peptides. The SIBLING proteins share many features such as gene location and structural characteristics (48, 50). The genes encoding the SIBLING proteins are all located within a 375,000 bp region on chromosome 4q21 (human) and display similar exon structures (50). It is likely that the SIBLING genes arose by gene duplication and divergence (49). Structural characteristics shared among the SIBLING proteins include the presence of an Arg-Gly-Asp (RGD) site which is important in cell-integrin binding, PTMs such as phosphorylation and glycosylation and high levels of acidic amino acids (51).

1.4.2.1 SIBLING Structure

Members of the SIBLING family possess a loose, flexible structure and this lack of structure has been postulated to be important for interacting with numerous binding partners including collagen, integrins and HA (49). NMR studies of OPN (49) and DPP (52) reveal the proteins to adopt a random coil conformation. Based on secondary-structure prediction programs, circular dichroism (CD) (53) and small-angle X-ray scattering (54), DMP-1 has also been shown to possess a random coil secondary structure. Of relevance, secondary structure programs predict MEPE to be an unstructured protein (55). Finally, BSP has been shown to be unstructured as will be discussed in greater detail later in this chapter.

1.4.2.2 Post-Translational Modifications of SIBLING Proteins
The SIBLING proteins are heavily modified by PTMs such as glycosylations and phosphorylations. Phosphorylations occur on serine, threonine and to a lesser extent, tyrosine residues. The kinases postulated to be responsible for phosphorylation are Golgi casein kinase (gCK), protein kinases (CK1 and CK2) and FAM20 (56). MS analysis of rat bone-extracted OPN show an average of 10 phosphate groups per molecule (57). DSP has between 6 to 10 phosphate groups (58) while DPP (comprising approximately 450 amino acid residues) is heavily phosphorylated with an average of 209 phosphates (59). The SIBLING protein DMP-1 exists as 37-kDa and 57-kDa peptides based on relative mobility on SDS-PAGE, containing 12 and 41 phosphates, respectively (60). Phosphorylation has been demonstrated to have a significant impact on the SIBLING proteins’ ability to modulate mineral formation. For example, phosphorylation of OPN is required for HA inhibition (57) while the phosphorylation of the 57 kDa fragment of DMP-1 alters its function from being a HA inhibitor to a HA nucleator (60). In dentin, Torres-Quintana et al. found that mineralization was decreased in the presence of kinase inhibitors, demonstrating the importance of these phosphorylations (61).

The SIBLING proteins also contain variable levels of glycosylation (e.g., rat bone-extracted OPN has four O-linked oligosaccharides); however, their precise functions are unknown.

1.4.2.3 SIBLING Function

The SIBLING proteins BSP, DMP-1 and DPP are thought to be HA nucleators whereas OPN and MEPE are thought to be inhibitors of HA. These proteins are expressed predominantly in mineralized tissues and are proposed to be important in
mineralization. Knock-out models of the various SIBLING proteins have been generated and all display abnormalities in bone and/or tooth formation.

At the early stages in life, there are observable bone phenotypes in BSP-deficient mice. At 0-4 months, the mice show decreased mineralization of cortical bones and shortened long bones, suggesting BSP is important in early mineral formation. DSPP-null mice displayed extensive tooth abnormalities similar to dentinogenesis imperfecta type III, a condition characterized by enlarged pulp chambers, increased width of predentin zone, hypomineralization and pulp exposure (62). Skeletal abnormalities such as enlarged growth plates, as well as dentin wall hypomineralization, are evident in DMP-1 knock-out mice (63, 64). Increased mineral content and bone mass are present in OPN-deficient and MEPE-deficient mice. Collectively, these findings demonstrate the physiological importance of the SIBLING proteins for proper bone and tooth formation (23, 65).

1.4.3 Bone Sialoprotein

Bone sialoprotein (BSP) is an acidic non-collagenous phosphoprotein and a member of the SIBLING protein family. This acidic protein was first isolated as 25 kDa fragments from the cortical bone of cow by Herring in 1963 (66). However, due to difficulties in extracting the protein, it was not until 1983 that intact BSP was purified from fetal calf bone by Fisher et al., (67). BSP has also been isolated from pig (68), rat (69), human (70), rabbit (71) and chicken (72) (Figure 1.3). More recently, a BSP-like protein has been identified in amphibian (African clawed toad) and reptile (caiman) species (73).

Mammalian BSP contains approximately 327 amino acid residues, 16 of which are the signal peptide (Figure 1.3). BSP contains the following functional domains: a
hydrophobic region near the N-terminus (spanning residues 18-45) shown to bind to type I collagen (74) two glutamic acid-rich regions located in the middle of the molecule, which are responsible for HA binding and nucleation and a C-terminal RGD cell-binding motif (75). Of relevance, there is a highly conserved thrombin-cleavage site at Arg27-Tyr28 within the type I collagen binding region (unpublished data).

Analysis of the deduced amino acid sequences of BSP from different mammalian species shows a high level of conservation. Moreover, identity of up to 90% is observed within specific domains (i.e., the two glutamic acid-rich regions, the RGD motif and the collagen-binding domain) (74, 76). The high level of conservation of these domains among species implies preservation of function (Figure 1.4).

1.4.3.1 Secondary Structure of Bone Sialoprotein

BSP has been shown by NMR (49, 77), CD spectroscopy (6, 78) and small angle X-ray scattering (6) to possess a loose, flexible structure. Furthermore, after rotary-shadowing TEM, BSP appears as a monomer possessing a ~10 nm globular structure linked to an elongated thread-like structure (~25 nm) (78). Similar to other SIBLING proteins, it has been hypothesized that this lack of order allows BSP to simultaneously interact with proteins such as type I collagen (49).
Figure 1.3. Schematic of BSP functional domains.

BSP contains the following functional domains: type I collagen-binding region (residues 18-45), two glutamic acid-rich regions (which are important for HA binding and nucleation) and a RGD cell-binding motif. Within the type I collagen binding region, there is a thrombin cleavage site at Arg27-Tyr28 (insert). Post-translational modifications such as $N$- and $O$- linked glycosylations, phosphorylations and tyrosine sulfations are shown.
Figure 1.4 Sequence alignments for mammalian bone sialoprotein.

The amino-acid sequences were aligned to generate a consensus sequence. Residues that are identical in five of the six sequences are indicated by upper case letters in the consensus sequence, whereas residues identical in three or more sequences are indicated by lower case letters. Residues that are conserved in fewer than three sequences are indicated by a period. [Adapted from (76)]
1.4.3.2 Post-Translational Modifications of Bone Sialoprotein

BSP is heavily modified by PTMs which account for up to 30% of the molecular weight of native BSP. PTMs include N- and O- linked glycosylations, phosphorylations, tyrosine sulfations and transglutaminase cross-linking. In the absence of PTMs, BSP has a molecular weight of approximately 33-34 kDa (calculated from the amino acid sequence). However, as a result of the heterogeneity in PTMs that BSP undergoes, the molecular weight of individual BSP molecules is highly variable. Based on two separate MS studies of human bone-derived BSP, the mean mass was determined to be ~52.5 kDa (79) and ~49 kDa (80). In both studies, a broad peak width (~20 kDa) was observed in the mass spectrum, reflecting the heterogeneity in PTMs. Of relevance, based on SDS-PAGE, native BSP has an apparent molecular weight of 60-80 kDa.

To date, two sulfated tyrosine residues near the RGD sequence (Tyr 259 or 263 and Tyr 297 or 298) have been identified (79). Although it was initially hypothesized that these residues may modulate cell-attachment capabilities, Midura et al., found that both bone-extracted and rBSP (unmodified BSP) have comparable cell attachment capabilities (81). Moreover, blocking of sulfation with chlorate treatment did not alter cell attachment capabilities of bone-extracted BSP.

The mammalian BSP sequence has three putative sites for N-linked glycosylation (Asn-X-Ser/Thr) located in the middle of the protein, two of which are conserved across all mammalian species (82). In addition, there is a fourth putative site for N-linked glycosylation in human BSP near the N-terminus (83). Matrix-assisted laser desorption mass spectrometry (MALDI-TOF MS) analysis of human bone-derived BSP has revealed 2 N-linked oligosaccharides (Asn 161 and Asn 166) and 11 O-linked oligosaccharides.
(Ser 92, 214 and 232 and Thr 95, 165, 167, 213, 214, 223, 230, 231 and 232) (84). In human bone-extracted BSP, Wutkke et al., identified only 8 of the 11 O-linked oligosaccharides and 4 N-linked oligosaccharides. Collectively, these studies demonstrate that glycosylation tends to be heterogeneous in both the level and sites of modification. Although the role of these glycosylations on BSP remains unclear, these modifications have no apparent affect on collagen-binding activity (74).

Similar to other SIBLING proteins, BSP is highly phosphorylated, particularly at serine and threonine residues. The mammalian BSP sequence has 5 putative serine phosphorylation sites for protein kinase C, 9-11 putative Ser/Thr phosphorylation sites for casein kinase II and 1-2 putative Tyr sites potentially for tyrosine kinase (76). A number of studies have been devoted to determining the quantity and location of the phosphorylation sites of BSP in vitro and in vivo (84-87). Findings from these studies demonstrate the heterogeneity of phosphorylations on BSP. Although phosphorylations on BSP have no apparent affect on collagen-binding activity (74), these phosphate groups are important in the modulation of mineral formation and this will be discussed below

1.4.3.3 Tissue Expression of Bone Sialoprotein

BSP expression is primarily restricted to mineralized tissues such as bone, dentin, cementum, enamel and mineralizing cartilage (5, 83, 88-91). However, other studies have described BSP to be present in non-mineralized areas such as trophoblastic cells of the placenta (5, 92), salivary glands (93) and platelets (94). Transcript levels for BSP are higher in newly formed bone and cementum than in other tissues (95, 96). However, maximal levels of BSP transcripts and expression are seen during embryonic bone formation. BSP has been shown to be concentrated at the mineralization front and cement
lines (boundary of an osteon) (97). Interestingly, by immunogold labeling, BSP has been shown to be associated with collagen fibres in mineralized tissues (98).

In pathological conditions, BSP is positively correlated with cancers that metastasize to bone such as breast (99, 100) and prostate cancers (101, 102). In addition, BSP has been shown to be associated with a 97 kDa protein produced by *Staphylococcus aureus*, a bacterium that infects bones and joints (103).

**1.4.3.4 Functions of Bone Sialoprotein**

BSP is implicated in a variety of physiological functions including cell attachment and signaling, HA binding and nucleation and type I collagen binding (76).

BSP has a highly conserved RGD site important for cell/integrin binding. The RGD site interacts with the αvβ3 integrin, a cell-surface receptor present in osteoclasts (104) and osteoblasts (105). The RGD sequence has significant function because when it is mutated to KAE, the cell/integrin binding of osteoblasts (106), chondrocytes (107) and fibroblasts (105, 108) is abolished. Binding of BSP to osteoblasts promotes osteoblast differentiation and mineralized nodule formation (109, 110), while binding of BSP to osteoclast progenitor cells appears to be involved in osteoclast generation (111, 112).

BSP is a potent nucleator of HA crystals in agarose (33), gelatin (113) and collagen gels (114). There is a strong interaction between BSP and HA with the $K_D$ of approximately 0.85 µM (115). The two glutamic acid-rich regions (with contiguous glutamic acid residues) of BSP are essentially responsible for the nucleation activity (6, 116). Both bone-extracted BSP and prokaryotic-expressed recombinant BSP (rBSP) lacking PTMs were capable of nucleating HA; however, rBSP was much less potent
(100-fold decrease in nucleation potency) (6). This indicates that PTMs are not essential, but aid in nucleation.

Initially, it was believed that electrostatic interactions were responsible for BSP binding to collagen since type I collagen has a slightly positive charge at neutral pH (98). However, our previous work demonstrated electrostatic interactions are only partly involved in the BSP-type I collagen interaction as binding to collagen was reduced, but not abolished, by the addition of cations, increase in ionic strength or decrease in pH (74). The BSP-collagen interaction is primarily hydrophobic in nature, as the majority of collagen-bound BSP was eluted by acetonitrile (114). Bone-extracted BSP and rBSP (unmodified BSP) bound to type I collagen with approximately equal affinity, $K_D = 12.1$ nM and $K_D = 14.2$ nM, implying PTMs are not critical for binding.

Using a solid-phase binding assay and synthetic peptides, the type I collagen-binding site and the structural aspects of collagen involved in its interaction with BSP were determined. The collagen-binding region on BSP was mapped to residues 18-45, a region rich in hydrophobic and basic amino acids and highly-conserved among species (74). Interestingly, previous studies from our laboratory have shown that thrombin cleaves rat BSP at Arg27-Tyr28, and abolishes collagen binding (114, 117). Moreover, BSP (114) displayed comparable binding affinities to monomeric and fibrillar type I collagen ($K_D = 13$ nM), but not to heat-denatured collagen (gelatin) with a binding affinity ($K_D$) of 44 nM. However, the non-helical telopeptides are not involved in BSP-collagen interaction as pepsin treatment of collagen had no apparent effect. These findings suggest the BSP-type I collagen interaction requires a native triple-helical type I collagen structure, but does not require either collagen telopeptides or fibrils (114).
1.4.3.5 Binding of ECM Proteins to Type I Collagen

A number of ECM proteins other than BSP have been shown to bind to type I collagen. OPN binds type I collagen at a highly conserved region containing hydrophobic and acidic amino acid residues (118). The collagen-binding domain of DMP-1 has been identified to be two acidic-amino-acid-rich regions found at the C-terminal end of the protein and has a dissociation constant of 3.8 ±1.7 µM (119). A significant decrease in binding due to substitution of these acidic domains with neutral amino acids suggests that the interaction between DMP-1 and type I collagen is electrostatic in nature (119). By EM, DPP has been shown to bind primarily to the fibril surface at the “e” band, which is in the middle of the gap region (120). Using rotary-shadowing TEM, the DPP binding location on type I collagen has been mapped to approximately 210 nm from the N-terminal end of the collagen molecule. This binding region corresponds to DPP covering residues 698 to 750, a region corresponding to an overlap region of the collagen fibril rather than a gap region (121).

1.5 PURPOSE OF THE THESIS

The mechanism of biomineralization is currently unknown. In bone, it has been proposed that an acidic phosphoprotein of the ECM, when bound to type I collagen, will induce the nucleation of HA. BSP is an acidic non-collagenous phosphoprotein that is expressed primarily in mineralized tissues and has been shown to be a potent nucleator of HA (6, 7). BSP is secreted into the ECM by osteoblasts, where it is hypothesized that it binds to type I collagen in the gap regions or the regions adjacent to it where early mineral formation occur. The collagen-binding region on BSP, rich in only hydrophobic and basic amino acids, spans residues 18-45, and is highly conserved among species.
However, the BSP-binding region on type I collagen is unknown. The first objective of this thesis is to determine the BSP-binding region on type I collagen using a chemical cross-linking method and rotary-shadowing TEM. These methods will be discussed in greater detail in the next chapters.

Within the collagen-binding region, there is a highly conserved thrombin cleavage site at Arg27-Tyr28 and, upon cleavage, collagen-binding is abolished. The physiological relevance of this conserved thrombin-cleavage site has, however, not been fully elucidated. In addition to HA nucleation, BSP has been demonstrated to function as a signaling molecule in physiological pathways. BSP contains a highly-conserved C-terminal RGD site that interacts with the αvβ3 integrin and has been shown to be important in cell adhesion and migration of osteoblast and tumour cells. Since BSP has been shown to be a chemotactic agent, we hypothesize that upon bone injury, thrombin becomes activated, then cleaves and releases BSP into the ECM. Subsequently, BSP can act as a signaling molecule to recruit osteoprogenitor cells, which will differentiate into osteoblasts. The second objective of this thesis is to determine whether thrombin cleaves and releases BSP from type I collagen. The thrombin digestion reactions will be analyzed using SDS-PAGE, western blotting and MALDI-MS.

Collectively, the results from these studies are important in understanding the initial stages of bone mineralization which may ultimately lead to the generation of therapeutic agents to promote bone repair.
1.6 REFERENCES


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CHAPTER TWO
2.1 INTRODUCTION

A variety of approaches including electron microscopy, synthetic triple-helical peptides, cyanogen bromide cleavage and chemical cross-linking have been utilized by researchers in locating the binding site(s) of various proteins on type I collagen. Chemical cross-linking appears to be a useful tool for mapping the BSP-binding site on collagen for the reasons discussed below.

Chemical cross-linking is an important tool for studying protein-protein interactions as it allows the formation of a covalent attachment between two interacting proteins (1). The two chemical cross-linkers selected for studying the BSP-collagen interaction in this study were \( \rho \)-azidophenacyl bromide (APB) and N-[4-(\( \rho \)-azidosalicylamido)butyl]-3’-(2’-pyridylthio) propionamide (APDP). Both are heterobifunctional cross-linkers possessing two different functional groups separated by a spacer arm. APB has a bromoacetyl group, whereas APDP contains a pyridyl disulfide. These functional groups are electrophilic, which makes them prone to react with the cysteine residues (strong nucleophiles) found in the genetically modified single-cysteine rBSP (1-100) peptides. The second functional group is a photoactivable group that non-specifically inserts or adds to carbon-hydrogen or nitrogen-hydrogen bonds upon UV exposure (1). It is disadvantageous for the cross-linkers to have this non-specific photoactivable functional group because the type I collagen region that is in close proximity in the BSP-cross-linker complex is unknown. The primary difference between the chemical cross-linkers lies in the length of the spacer arm. The spacer arm of APDP is approximately 2.1 nm (2) while APB has a spacer arm less than 1.2 nm in length (3). The longer spacer arm of APDP may potentially interact with preferred amino acid residues
on type I collagen that are further away from the BSP-collagen binding site and therefore may increase the chances of forming BSP-type I collagen cross-linked products. In addition, chemical cross-linking should not disrupt the triple-helical type I collagen structure that is essential for binding to BSP (4). Furthermore, when chemical cross-linking is coupled with mass spectrometry (MS), the precise amino acid sequences involved in the interactions can be determined.

The purpose of the present study is to use a chemical cross-linking method to map the BSP-binding site on collagen. These experiments involve five single-cysteine rBSP (1-100) mutants generated by site-directed mutagenesis, type I collagen and the chemical cross-linkers: APB and APDP. Multiple approaches were taken to maximize the efficiency of cross-linking and recovery of BSP-type I collagen cross-link products.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

For protein expression and purification, E.coli (DE3) cells were purchased from EMD Biosciences/Novagen (Gibbstown, NJ). Medical grade N₂ was obtained from Praxair Inc. (Mississauga, ON). Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich (St. Louis, MO). Urea UltraPure grade was from BioShop Canada (Burlington, ON).

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): ammonium persulfate (APS), N, N, N’, N’-tetramethylethane-1, 2-diamine (TEMED), acrylamide/bis-acrylamide, SDS and pre-stained protein standards were all purchased from Bio-Rad Laboratories (Hercules, CA). β-mercaptoethanol and glycine were obtained from Thermo Fisher Scientific (Rockford, IL). Isopropanol was from VWR
International (West Chester, PA). Imidazole, Stains-All and silver nitrate were all purchased from Sigma-Aldrich.

For chemical cross-linking, the cross-linkers: APDP was purchased from Thermo Fisher Scientific while APB was purchased from Sigma-Aldrich. The reducing agent, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was from Sigma-Aldrich. For dissolving the chemical cross-linkers, dimethylformamide, (DMF) and methanol were obtained from VWR International. Type I collagen was extracted from rat tail tendons in our laboratory as previously described (5).

2.2.2 METHODS

2.2.2.1 Expression and purification of rBSP (1-100) mutants

A schematic diagram of BSP depicting the amino acids mutated to cysteine is shown in Figure 2.1. The plasmids of the five single-cysteine mutants were generated by site-directed mutagenesis by Aaron Langdon (past MSc student in Drs. Goldberg and Hunter’s Laboratories). Three of the five single-cysteine rBSP (1-100) mutants (S16C, Y31C and Y24C) were present in adequate amounts in the laboratory for the chemical cross-linking experiments while the remaining two single-cysteine mutants (F36C and S49C) were expressed and purified as described below.

The two single-cysteine rBSP (1-100) mutants (F36C and S49C) contain a penta-His fusion tag at the C-terminus and were transformed into E.coli BL21 (DE3) cells and grown in Luria broth (LB) containing 15 µg/ml kanamycin. When the optical density of the cells reached 0.6 – 0.8, the cells were induced with 2 mM IPTG and grown for an additional 4 hours. Sonication in His-column binding buffer (5 mM imidazole, 0.5 M NaCl, 0.02 Tris-HCl, 6 M urea, pH 7.9) was used to lyse the bacterial cells.
Subsequently, the cell lysate was fractionated by centrifugation and the supernatant recovered.

The single-cysteine rBSP (1-100) mutants were purified extensively. The first purification step was nickel affinity chromatography. The cell extract was loaded onto Poly-prep chromatography columns (Bio-Rad) packed with 1.5 ml His-bind resin (Novagen) that had been previously charged with 50 mM NiSO₄. The columns were first washed with 10 column volumes of binding buffer and the proteins eluted with 500 mM imidazole-containing elution buffer (binding buffer containing 500 mM imidazole). The eluted fractions were separated on 12.5% polyacrylamide gels using the Phastgel system (Amersham Biosciences) and the gels stained with Stains-All (30 mM Tris-HCl pH 8.0, 7.5% formamide, 25% isopropanol, and 0.0025% Stains-All) and silver nitrate as previously described (6).

The protein-enriched fractions from nickel affinity were pooled and diluted with Mono A buffer (50 mM Tris-HCl, 7M urea, pH 7.4) and purified further by ion-exchange chromatography. The fractions were loaded onto a Q Sepharose Fast Flow 1 x 10 cm column (Fast Q) connected to the Fast Protein Liquid Chromatography (FPLC) system (AKTA-purifier Amersham Pharmacia Biotech). Proteins were eluted with a linear gradient of sodium chloride (0 – 500 mM) at a flow rate of 2.0 ml/min. Fractions of 1 ml were collected and selected fractions stained with Stains-All and silver. Fractions containing the peptide of interest were pooled and concentrated to a volume of approximately 2.5 ml using a pressurized Amicon ultrafiltration apparatus with an YM-3 (3-kDa cutoff) membrane (Millipore).
The concentrated protein-enriched fractions were purified further by gel filtration using a Superdex 200PG column (1.6 cm x 60 cm) (Amersham Biosciences) in 50 mM Tris, 4 M urea, 200 mM NaCl, pH 7.4. The fractions containing the protein of interest (determined by SDS-PAGE) were dialyzed using 10 mM ammonium bicarbonate for 72 hours at 4°C using a 3-kDa cutoff Spectra/Por dialysis membrane (Spectrum Laboratories). After dialysis, the fractions were aliquoted and lyophilized. Finally, the purity and quantity of the protein samples were determined by Bicinchoninic acid (BCA) assays, amino acid analysis (Advance Protein Technology Centre, The Hospital for Sick Children, Toronto) and MALDI mass spectrometry (Bruker® Reflex IV MALDI TOF MS, Department of Biochemistry, UWO, London).
Figure 2.1 Schematic diagram of BSP depicting the amino acids mutated to cysteine. BSP contains approximately 300 amino acid residues (as indicated by the numbers). The amino acids mutated to cysteine for chemical cross-linking are highlighted in orange. The selected amino acids are within and surrounding the collagen-binding region.
2.2.2.2 Chemical Cross-Linking with APB and APDP and Purified using TALON® His₆-Tag Cobalt Resin

A general overview of the chemical cross-linking protocol is shown in Figure 2.2. Chemical cross-linking reactions between single-cysteine rBSP mutants (S16C, Y24C, Y31C, F36C and S49C) and type I collagen were carried out using heterobifunctional cross-linkers APB and APDP. Additionally, control cross-linking reactions between wild-type (WT) rBSP (1-100) and type I collagen using APB and APDP were performed. These reactions served as the control as WT rBSP (1-100) does not contain a cysteine residue that is required for cross-linking to occur.

First, bond-breaker solution (9 mM TCEP in 2x PBS) was added to single-cysteine BSP (1-100) mutants (1 mg/mL dissolved in 2x PBS, 3 mM EDTA pH 7.5) and incubated for 30 min. Subsequently, the bond-breaker solution was removed by buffer exchange using Amicon® Ultra-0.5 centrifugal filter units. The single-cysteine BSP mutants were incubated with one of the chemical cross-linkers: 50 mM APDP in DMF or 50 mM APB in methanol for 30 min in the dark. Excess cross-linkers were then removed by buffer exchange using Amicon® Ultra-0.5 centrifugal filter units. Type I collagen (1 mg/mL) in 5 mM acetic acid was then added to the reaction mixture and incubated in the dark for 1 hour. To induce chemical cross-link formation, the reaction mixture was irradiated with a UV ultraviolet lamp (Thermo Scientific Pierce) at a wavelength of 365 nm for APDP and at 254 nm for APB for 5 min.

In order to maximize the recovery of successful BSP-type I collagen cross-link products, reactions were purified using TALON® His₆-Tag cobalt resin (Clontech Laboratories) and concentrated using Amicon® Ultra-4 centrifugal filter units and Amicon® Ultra-0.5 centrifugal filter units. The reactions were loaded onto a 6% SDS-
PAGE gel in the absence of a reducing agent and run at 80 V until the separating gel was reached where the voltage was increased to 120 V. The gels were then stained with Stains-All and silver (6) for analysis.
Figure 2.2 An overview of the chemical cross-linking protocol. Chemical cross-linking reactions between single-cysteine rBSP (1-100) mutants and type I collagen were carried out using the cross-linkers APB or APDP. The chemical cross-linking reactions were purified using a His$_6$–Tag cobalt resin or nickel magnetic beads and then concentrated using centrifugal filter devices. The products of the cross-linking reactions were then analyzed using Stains-All and silver.
2.2.2.3 Chemical Cross-Linking using APDP and Purification using Millipore PureProteome\textsuperscript{TM} Nickel Magnetic Beads

In hopes to increase the recovery of successfully cross-linked BSP-type I collagen complexes, the purification resin was changed from the TALON\textsuperscript{®} His\textsubscript{6}-Tag cobalt resin (Clontech Laboratories) to nickel magnetic beads (Millipore PureProteome\textsuperscript{TM}).

Chemical cross-linking reactions between single-cysteine BSP mutants (S49C and F36C) and type I collagen were carried out using APDP as previously described. The single-cysteine mutants, S49C and F36C, were used for this experiment as these two mutants showed higher levels of potential cross-links over the other single-cysteine mutants. Additionally, the cross-linker APDP was used rather than APB for the same reasons.

After chemical cross-linking, the reactions were purified using an aliquot of 200 µL of nickel magnetic beads present in a 1.5 mL microcentrifuge tube. The magnetic beads were first equilibrated with 500 µL of equilibration buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8) for 1 min at 22°C. The equilibration buffer was removed by placing a small circular magnet (Staples\textsuperscript{®}, Canada) at the side of the microcentrifuge tube, resulting in the magnetic beads adhering to the side of the tubes allowing for the buffer to be easily removed by a pipette. The protein sample was then added to the magnetic beads and incubated for 1 hour at 22°C. Subsequently, five consecutive washes using the wash buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8) were performed to remove any unbound proteins. Elution of the protein was done by adding an aliquot of 100 µL of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole, pH 8) and incubation for 2 min at 22°C. The eluted fraction was collected with use of the magnet and the recovered solutions concentrated.
and analyzed by SDS-PAGE and staining with Stains-All and silver (as previously described) for analysis of potential BSP-type I collagen cross-links.

2.2.2.4 Characterization of the Additional Bands after Chemical Cross-Linking

To determine whether the high-molecular-weight bands present after chemical cross-linking are rBSP (1-100) cross-linked to itself, cross-linking reactions were carried out with the single-cysteine rBSP (1-100) F36C mutant and type I collagen using APDP as previously described. The single-cysteine rBSP (1-100) F36C mutant was chosen for this experiment as this mutant showed high levels of the high-molecular-weight bands after cross-linking. Prior to loading the samples onto a 6% SDS-PAGE gel, the samples were heated to 95°C in the presence or absence of a reducing agent (β-mercaptoethanol) for 10 min. In the case where the bands are a result of the single-cysteine rBSP (1-100) mutants self-cross-linking, the disulfide bonds will break in the presence of β-mercaptoethanol and the polymeric BSP will be reduced to monomeric rBSP (1-100) peptides. The gel was then stained with Stains-All and silver for analysis. Additionally, to ensure that the sensitivity and detection of type I collagen is not decreased with Stains-all and silver, a second 6% SDS-PAGE gel was stained with only silver nitrate (6).

2.2.2.5 Chemical Cross-Linking using APDP and Purification using Gel Filtration and Millipore PureProteome™ Nickel Magnetic Beads

Another attempt to increase the recovery of successful BSP-type I collagen cross-linked polymers was to use two purification approaches: gel filtration and nickel magnetic beads. Chemical cross-linking reactions between the single-cysteine BSP mutant (S49C) and type I collagen were carried out using APDP as previously described. Prior to purification of the cross-linking reactions using nickel magnetic beads, the reaction mixture was purified by gel filtration on a Superdex 200PG column. This step
should separate the lower-molecular-weight single-cysteine rBSP (1-100) mutants that are not cross-linked to collagen from larger-molecular-weight cross-linked polymers. The removal of the potentially competing binding ligand for the nickel resin should enhance binding and recovery of rBSP-type I collagen cross-linked products. Furthermore, the higher-molecular-weight fractions containing the co-eluting unbound type I collagen should not bind to the nickel resin since it does not contain the His$_6$).

2.3 RESULTS

2.3.1 Expression and purification of rBSP (1-100) mutants

Two single-cysteine rBSP (1-100) mutants (F36C and S49C) were overexpressed in *E. coli* BL21 (DE3) cells and purified extensively using three purification columns. The first purification step was nickel affinity chromatography. The eluted fractions were separated on 12.5% polyacrylamide gels and stained with Stains-All and silver to determine the purity of the fractions (results not shown).

The protein-enriched fractions from nickel affinity were pooled and further purified by ion exchange using a Fast Q column connected to the FPLC system. The presence of proteins was monitored spectrophotometrically at 230 and 280 nm (Figure 2.3A). Selected fractions were separated on 12.5% polyacrylamide gels and stained with Stains-All and silver for analysis (Figure 2.3A, insert).

The protein-enriched fractions from ion exchange were pooled and loaded onto a Superdex 200PG column and monitored spectrophotometrically at 230 and 280 nm for the presence of protein (Figure 2.3B). The fractions containing the protein of interest (Figure 2.3B, insert) were dialyzed against 10 mM ammonium bicarbonate and then aliquoted and lyophilized.
The purity of the protein samples were further verified by MALDI mass spectrometry. The single-cysteine rBSP (1-100) F36C mutant with a 5xHis tag was detected by MALDI mass spectrometry as a single peak at 12,029 Da. The theoretical mass based on the known amino acid sequence is 12,036 Da. Additionally, the quantity of the protein samples were determined by BCA assays (data not shown).
The protein-enriched fractions from nickel affinity were pooled and diluted with Mono A buffer (50 mM Tris-HCl, 7 M urea, pH 7.4) and loaded onto a (A) Fast Q packed column connected to the FPLC system. Proteins were eluted with a linear gradient of 0-0.5 M NaCl. Fractions of 1 mL were collected and selected fractions (green box) were purified further with a (B) Superdex 200PG gel filtration column. The fractions containing the protein of interest were pooled and lyophilized (green box). The absorbance at 280 nm is in blue and the absorbance at 230 nm is in red.
2.3.2 BSP-type I collagen cross-linking reactions using APB

Chemical cross-linking between the five single-cysteine rBSP (1-100) mutants was carried out using APB. Subsequently, type I collagen (1 mg/ml) in 5 mM acetic acid was then added to the reaction mixture. The products of the cross-linking reactions were loaded onto a 6% SDS-PAGE and analyzed by Stains-All and silver (Figure 2.4).

The band corresponding to the single-cysteine rBSP (1-100) mutants appears near 18 kDa on SDS-PAGE, even though the actual molecular weight (based on the known amino acid sequence) is approximately 12.2 kDa. With regards to type I collagen, the molecular weight of each collagen α-chain is approximately 97 kDa (based on the known amino acid sequence), however; based on SDS-PAGE, the α₁ and α₂ chains migrate much slower than expected: at approximately 120 kDa and 110 kDa, respectively. From the known molecular weights of the rBSP (1-100) mutants and the collagen α-chains, a successful cross-link would be approximately 110 kDa.

However, due to the slow migration of rBSP (1-100) mutants and collagen α chains, the successful BSP-type I collagen cross-link would be in the range of 128 kDa to 138 kDa. Therefore, a band corresponding to a successful cross-link BSP-collagen product would be immediately above the α₁ and α₂ chains of type I collagen (Figure 2.4; the lanes labeled “coll” indicate the position of the α₁ and α₂ chains). There appears to be a band present, albeit weakly-stained, at approximately 130 kDa that may correspond to a BSP-collagen cross-link (Figure 2.4; as indicated by the purple arrow). Furthermore, this possible cross-link at 130 kDa was not observed in the control cross-linking reactions using rBSP (1-100). However, there are numerous additional bands above and below 130 kDa. As well, the generally weakly stained band suggests recovery of the cross-linked protein is low and/or the cross-linking efficiency is low.
Figure 2.4 Analysis of BSP-type I collagen cross-linking reactions using APB.

Chemical cross-linking reactions between single-cysteine BSP mutants (S16C, Y24C, Y31C, F36C and S49C) and type I collagen were carried out using APB. Reactions were purified using TALON® His$_6$-Tag cobalt resin and concentrated using Amicon® Ultra-4 centrifugal filter units and Amicon® Ultra-0.5 centrifugal filter units prior to loading onto a 6% SDS-PAGE gel and stained with Stains-All and silver for analysis. Potential cross-links between BSP and collagen are present at approximately 130 kDa (purple arrow). “M” indicates the molecular marker; rBSP refers to the wild type peptide.
2.3.3 BSP-Type I Collagen Cross-Linking Reactions using APDP

An attempt was made to increase the cross-linking efficiency between BSP and type I collagen by using APDP (2.1 nm), which contains a longer spacer arm than APB (1.2 nm).

Once again, there appears to be a band, albeit weakly stained, at approximately 130 kDa corresponding to a potential BSP-collagen cross-link (Figure 2.5; as indicated by the purple arrow). Furthermore, this possible cross-link at 130 kDa was not observed in the control cross-linking reactions using rBSP (1-100). However, the same issues are evident: there are additional bands above and below the potential cross-linked product. Additionally, the weakly stained band suggests recovery of the cross-linked protein is low and/or the cross-linking efficiency is low.
Figure 2.5 Analysis of BSP-type I collagen cross-linking reactions using APDP.

Chemical cross-linking reactions between single-cysteine rBSP mutants (S16C, Y24C, Y31C, F36C and S49C) and type I collagen were carried out using APDP. Reactions were purified using TALON® His$_6$-Tag cobalt resin, concentrated by centrifugal ultrafiltration, and analyzed by 6% SDS-PAGE and staining with Stains-All and silver. Potential cross-links between BSP and collagen are present at approximately 130 kDa (purple arrow). “M” indicates the molecular-weight marker.
2.3.4 BSP-Type I collagen Cross-Linking Reactions using APDP and Purified using Millipore PureProteome™ Nickel Magnetic Bead

In hopes to enhance the cross-linking efficiency, the purification resin was changed from the TALON® Histag cobalt resin to Millipore PureProteome™ nickel magnetic beads. Chemical cross-linking reactions between single-cysteine rBSP mutants (S49C and F36C) and type I collagen were carried out using APDP as previously described.

Unfortunately, no increase in the recovery of the cross-linked products was observed (as indicated by the weakly stained band at approximately 130 kDa; Figure 2.6). As well, there are additional bands above and below the potential BSP-type I collagen cross-link present, suggesting this purification resin did not increase the recovery of the cross-linked products in the expected manner.

2.3.5 Characterization of the Additional Bands Present after Cross-Linking

To determine whether the additional bands above and below the potential BSP-collagen cross-link in Figures 2.4 and 2.5 are the result of the single-cysteine rBSP (1-100) mutants cross-linking to itself, chemical cross-linking reactions with F36C, WT rBSP (1-100) and type I collagen were performed using APDP. Prior to loading the samples onto a 6% SDS-PAGE gel, the samples were heated to 95°C in the presence or absence of a reducing agent (β-mercaptoethanol) for 10 min. The gels were stained with Stains-All (Figure 2.7A), the combined Stains-All silver protocol (Figure 2.7B) or only silver (Figure 2.7C).

The additional bands, above and below the potential cross-linked product (Figure 2.7A and 2.7B yellow box) from samples not treated with β-mercaptoethanol, were not
observed when β-mercaptoethanol was added (Figure 2.7A and 2.7B; green box). As well, the blue smear present with Stains-All in the absence of β-mercaptoethanol is indicative of an acidic protein (Figure 2.7A). These findings suggest the bands are a result of the single-cysteine rBSP (1-100) mutants cross-linking to itself.

The 6% SDS-PAGE stained with silver nitrate alone (Figure 2.7C), ensured that sensitivity and detection of type I collagen is not decreased with Stains-All and silver. No apparent differences are observed between the two staining protocols.
Chemical cross-linking reactions between single-cysteine BSP mutants (S49C and F36C) and type I collagen were carried out using APDP. Reactions were purified using the nickel magnetic beads (Millipore PureProteome™) and concentrated by centrifugal ultrafiltration prior to loading onto a 6% SDS-PAGE gel and staining with Stains-All and silver for analysis. “M” indicates the molecular marker.
Figure 2.7 Characterization of the additional bands present after cross-linking. Chemical cross-linking was carried out using F36C, WT rBSP (1-100) and type I collagen using APDP to determine whether the bands observed in (Figures 2.4 and 2.5) are the result of single-cysteine rBSP mutants self-cross-linking. Prior to loading the samples onto a 6% SDS-PAGE gel, the samples were heated to 95°C in the presence or absence of β-mercaptoethanol. The gels were then stained first with (A) Stains-All and followed by (B) silver for analysis. The bands that appear as a blue smear (yellow box) are the result of single cysteine rBSP (1-100) F36C mutant cross-linking to itself and are reduced to monomeric rBSP (1-100) F36C in the presence of β-mercaptoethanol (green box). Identical samples were electrophoresed on a second 6% SDS-PAGE gel and stained with silver only (C). “M” indicates the molecular marker.
2.3.6 BSP-type I collagen cross-linking reactions using APDP and purified using gel filtration and nickel magnetic beads

In order to increase the recovery of successful BSP-type I collagen cross-linked polymers, two purification approaches were used: gel filtration and nickel magnetic beads. Chemical cross-linking reactions between the single-cysteine BSP mutant (S49C) and type I collagen were carried out using APDP as previously described. Prior to purification of the cross-linking reactions using nickel magnetic beads, the reaction mixture was separated by gel filtration on a Superdex 200PG column. This step separated the single-cysteine rBSP (1-100), and the smaller rBSP (1-100) polymers that were not cross-linked to collagen from larger molecular weight cross-linked polymers. The fractions consistent with containing BSP-type I collagen cross-links were collected (Figure 2.8A, orange box), and purified further using the nickel magnetic beads. Unfortunately, from the gel electrophoresis analysis, no increase in the efficiency of the cross-linked products was observed using this approach (Figure 2.8B).
Figure 2.8 BSP-type I collagen cross-linking reactions using APDP and purification by gel filtration and nickel magnetic beads.

Chemical cross-linking reactions between the single-cysteine BSP mutant (S49C) and type I collagen were carried out using APDP. The cross-linking reactions were purified by gel filtration and nickel magnetic beads. (A) Gel filtration on a Superdex 200PG column. The fractions potentially containing BSP-type I collagen cross-links were collected (A; orange box) and purified further using nickel magnetic beads. The purified samples were then loaded onto a 6% SDS-PAGE gel and stained with (B) Stains-All and silver for analysis. “M” indicates the molecular-weight marker.
2.4 DISCUSSION

Chemical cross-linking is a good protocol for mapping the BSP-binding region on type I collagen as it allows the formation of a covalent attachment between two interacting proteins. A major advantage is that when coupled with MS it facilitates the determination of the BSP-binding site on collagen to the amino-acid level. Once the covalent cross-linked product is characterized by MS, our goal would have been to use molecular modeling (MM) to simulate the BSP-type I collagen interaction and to pinpoint the interacting amino acids (7).

Multiple approaches were taken to maximize the efficiency and/or recovery of successful BSP-type I collagen cross-linked products. However, the efficiency and/or recovery of the cross-linked products observed from the gels stained with Stains-All and silver were unexpectedly low in all instances and therefore the complexes were not further characterized.

2.4.1 Single-cysteine rBSP (1-100) mutants

To maximize the chances of a successful BSP-type I collagen cross-link, five single-cysteine mutants were generated. The residues selected for cysteine substitutions are within and surrounding the type I collagen-binding domain in BSP. Within the collagen-binding region, the residues selected for cysteine substitution (Y24, Y31 and F36) are bulky, aromatic residues and were chosen in order to minimize steric hindrance between rBSP (1-100) peptides and collagen in the presence of cross-linker agents. Residues surrounding the collagen-binding domain were selected in case the mutations within the collagen-binding region interfered with the BSP-collagen interaction. The residues selected for cysteine substitution (S16 and S49) had chemically similar side-
chains as steric hindrance was less of a consideration. Additionally, the selected residues on BSP were mutated to cysteine as BSP does not contain any cysteine residues and therefore it is known where on the protein the cross-linker would be bound.

2.4.2 BSP-type I collagen cross-linking reactions using APB and APDP

There was a possible BSP-collagen cross-link at approximately 130 kDa when the cross-linkers APB and APDP were used. The presence of weakly stained bands suggests the cross-linking efficiency and/or recovery of the cross-linked products is low. This was unexpected as the chemical cross-linkers APB and APDP have been used in many cross-linking experiments studying protein-protein interactions. APB has been utilized to identify the binding sites within the $b$ subunit of *E.coli* ATP synthase that are responsible for interacting with $\alpha$, $\beta$, and $\gamma$ subunits (8). APB was also used to study the interaction between yeast cytochrome $c$ and cytochrome $c$ peroxidase (9). With regards to APDP, this cross-linker has been utilized by Yasui *et al.* to study the interaction between a pigment epithelium-derived factor (PEDF) and type I collagen (10).

In addition, multiple steps were taken to maximize the cross-linking efficiency and recovery of the successful BSP-collagen cross-linked complex. Following the reaction of APB or APDP with single-cysteine rBSP (1-100) mutants, excess cross-linkers were removed by buffer exchange. This step was carried out to reduce non-specific cross-link formation. Additionally, following UV exposure to induce cross-link formation, the reactions were affinity purified and concentrated. These two steps were carried out to maximize the recovery of successful BSP-type I collagen cross-link products.
It is also puzzling that there are unbound $\alpha_1$ and $\alpha_2$ collagen chains present since the cross-linking reaction products were affinity purified to recover the poly-His-containing proteins. A potential explanation for this is that collagen bound non-specifically to the purification resin. Therefore a different purification protocol was used, as discussed below.

**2.4.3 Chemical cross-linking using APDP and purification using nickel magnetic beads**

Using nickel magnetic beads we observed no increase in the amount of the BSP-collagen cross-linked products (Figure 2.6). This was unexpected since the nickel magnetic beads have been shown to achieve high purity and yield of His$_6$-tag proteins (11, 12). The single-cysteine mutants, S49C and F36C were used for this experiment because they showed higher levels of potential cross-links over the other single-cysteine mutants. Additionally, the cross-linker APDP was used rather than APB for the same reasons. However in spite of all the modifications, cross-linked product was not evident in high yield. A potential explanation of why this purification resin did not enhance the recovery lies in the additional bands present after chemical cross-linking.

**2.4.4 Characterization of the additional bands present after cross-linking**

Since the additional bands (in the absence of $\beta$-mercaptoethanol) were not observed when $\beta$-mercaptoethanol (disulfide bond ‘breaker’) was added (Figure 2.7A and 2.7B; green box), these bands are likely a result of the single-cysteine rBSP (1-100) mutants cross-linking to itself. Additional evidence that the bands are a result of the single-cysteine rBSP (1-100) mutants cross-linking to itself is the blue staining of these higher molecular weight bands with Stains-All, which is indicative of an acidic protein.
Since there was a concern that excess rBSP peptide, both unreacted and cross-linked to itself, would interfere with the rBSP-collagen product binding to the nickel affinity column, a strategy to remove the majority of the unwanted peptide was developed.

2.4.5 BSP-type I collagen cross-linking reactions using APDP and purified using gel filtration and nickel magnetic beads

No increase in the amount of the higher molecular weight recovered cross-linked products was observed when the single-cysteine rBSP (1-100) mutants and smaller polymeric BSP that are not cross-linked to collagen were first removed by gel filtration (Figure 2.8B). However, the low level of BSP-collagen cross-linked products observed is not likely due to the cysteine residues being unreactive. Previous work with fluorescein-5-maleimide demonstrated that the cysteine residues found in the single-cysteine (1-100) rBSP peptides are available and able to react with the chemical cross-linkers (unpublished data). A potential explanation for the low BSP-collagen cross-linked products lie in the spacer arm, perhaps it was not long enough to reach a collagen amino acid from the cysteine residue of the single-cysteine rBSP (1-100) peptide. However, these two cross-linkers possess the longest spacer arms among commercially available heterobifunctional cysteine-reactive cross-linkers with a photoactivable group. For example, benzophenone-4-maleimide (BPM) has a spacer arm that is less than 0.9 nm. In addition, BPM has a photoactivatable group that has been shown to have a preference for methionine residues. This may potentially decrease the BSP-collagen cross-linked products formed as methionine residues are fairly spread out within collagen molecules (13). In order to increase the sensitivity of detection, some researchers have utilized trifunctional cross-linkers possessing a biotin group such as sulfo(succinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido] ethyl-1,3-dithiopropionate (sulfo-
SBED) (14, 15). Subsequently, the cross-linked products would be enriched through affinity chromatography. However, since the cross-linking efficiency is low; this approach would require a sufficiently large amount of protein in order to obtain the desired amount BSP-collagen cross-linked product. Therefore, due to these difficulties in obtaining sufficient BSP-collagen cross-linked product, an alternative protocol using rotary-shadowing transmission electron microscopy (rotary-shadowing TEM) was used to map the BSP-binding site on type I collagen (discussed in the next chapter).
2.6 REFERENCES


CHAPTER THREE
3.1 INTRODUCTION

Rotary-shadowing transmission electron microscopy (rotary-shadowing TEM) is an excellent tool for identifying protein binding sites on other proteins because it offers high-contrast visualization of individual macromolecules. This is made possible by evaporation of a contrast-enhancing metal (e.g. platinum) that produces a fine grain onto the specimens at a low angle (i.e. 5-15°) (1-3). Researchers have utilized rotary-shadowing TEM extensively for locating the type I collagen-binding site(s) of various proteins such as proteoglycans, dentin phosphophoryn (DPP) and integrins (4-7). For instance, rotary-shadowing TEM shows DPP, a proposed mediator of mineralization in dentin, binding to type I collagen at a distance that is approximately 210 nm from the N-terminal end of the collagen molecule. This corresponds to DPP covering residues 698 to 750 on collagen upon binding (an overlap region of the collagen fibril) (4). Using gold-labeling EM, San Antonio et al., mapped the heparin-binding location to the N-terminal region of collagen (5). This method has also been used to map the collagen-binding sites for integrins α₁β₁ and α₂β₁ (6). Similarly, decorin has been mapped to a region near the C-terminus, which corresponds to the c₁ band in the overlap region of the collagen fibril D-period (7). Collectively, these studies demonstrate rotary-shadowing TEM to be a promising tool for studies of protein interactions with collagen.

Since studies on chemical cross-linking of BSP to type I collagen were hampered because of low yield, rotary-shadowing TEM was used to visualize the BSP-binding site on type I collagen. Using this method, the structures of type I collagen (8) and BSP (9) have been successfully elucidated by others. Type I collagen appears semi-flexible and
rod-like, while BSP appears as a monomer with a 10-nm globular structure that is linked to an elongated thread-like structure of ~25 nm.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Type I collagen was either extracted from rat tail tendons [as previously described (10)], or obtained commercially from Sigma-Aldrich (St. Louis, MO). TEM copper grids, 400 mesh, and mica were from Electron Microscopy Sciences (Hatfield, PA). Ammonium formate and acetic acid were purchased from Sigma-Aldrich. Glycerol was from EMD Biosciences/Novagen (Gibbstown, NJ). Carbon rods and platinum were purchased from SPI Supplies (West Chester, PA).

3.2.2 METHODS

3.2.2.1 Rotary-shadowing TEM

A general overview of the protocol is illustrated in Figure 3.1.

The interaction of BSP and type I collagen was examined using glycerol-spraying/low-angle rotary-shadowing TEM (2, 3) at The Microscopy Imaging Laboratory (University of Toronto). Full-length rBSP was dissolved in 70% glycerol/30% 0.01 M ammonium formate to a final concentration of 50 µg/ml. Type I collagen (1 mg/ml prepared in-house or 1 mg/ml obtained from Sigma) was stored in acetic acid, and dialyzed with 0.01 M ammonium formate, pH 7.2, prior to use. An anti-collagen I antibody (Sigma) used to identify the N-terminal end of the collagen molecules was diluted to 5 µg/ml in 70% glycerol/30% 0.01 M ammonium formate (v/v).

The samples prepared for rotary-shadowing TEM are outlined in Figure 3.2. An aliquot of 10 µl of each sample was mixed together in a microcentrifuge tube, vortexed
and incubated overnight at 4°C. A 20 µl aliquot of the sample mixture was then sprayed onto freshly cleaved mica sheets (Electron Microscopy Sciences) with a pressurized duster (Lyreco, Markham ON) at a distance of approximately 30 cm. In addition, the sample buffer (70% glycerol/30% 0.01 M ammonium formate) was rotary-shadowed and served as a control. Micas with the samples were then dried in vacuum and rotary-shadowed with platinum with an electron-bombarded source at an angle of 5°. Subsequently, the samples were backcoated at 90° with a carbon film. The films were then floated off the mica and transferred onto three 400-mesh TEM copper grids for analysis in a Philips CM-10 TEM at the UWO Biotron operating at 80 kV. The images were taken using the AMT Image software and measurements were made with the ImageJ software (NIH).
Figure 3.1 An overview of the rotary-shadowing TEM protocol.

Full-length rBSP (50 µg/ml) and type I collagen (1mg/ml) were incubated overnight at 4 °C and then sprayed onto freshly cleaved mica sheets. Mica containing the samples were then rotary-shadowed with platinum and backcoated with a carbon film. The films were then floated off the mica and transferred onto 400-mesh copper grids for TEM analysis.
**Figure 3.2 Sample preparations for rotary-shadowing TEM.**

The different sample preparations for rotary-shadowing TEM using rBSP (50 µl/ml), two sources of type I collagen (prepared in-house and purchased from Sigma), and a N-terminal type I collagen antibody (5 µg/ml) are shown.
3.3 RESULTS

3.3.1 Rotary-shadowing TEM of recombinant BSP

Rotary-shadowing TEM shows rBSP as a monomer possessing a 10-nm globular structure linked to an elongated thread-like structure of ~25 nm (Figure 3.3B). The schematic diagram in (Figure 3.3C) illustrates the shape of rBSP particles found in (Figure 3.3B), which is consistent with the literature (9).

3.3.2 Type I collagen rotary-shadowed with platinum

Two sources of type I collagen (prepared in-house and purchased from Sigma) were rotary-shadowed with platinum. The TEM images in (Figure 3.4) show representative type I collagen molecules observed upon examination of more than 50 collagen molecules (from multiple TEM copper grids) for each collagen source. The majority of the collagen molecules from Sigma (greater than 90%) appear semi-flexible and rod-like. Measurements of the collagen lengths with the ImageJ software reveal a mean of 270 nm and a standard deviation of 32 (at the 95% confidence level). In contrast, the lengths of the collagen molecules prepared in-house are quite varied. In addition to semi-flexible and rod-like collagen molecules, collagen molecules with lengths of 700 nm or greater are present, possibly indicative of aggregation.

3.3.3 N-terminal type I collagen antibodies rotary-shadowed with platinum

To determine the orientation of the type I collagen, antibodies recognizing the N-terminal end of type I collagen were used. The antibodies to type I collagen after rotary-shadowing appear as globular structures with diameters of approximately 10 nm (Figure 3.5).
3.3.4 Binding of rBSP on type I collagen

Our initial TEM studies of the rBSP-collagen interaction involved the use of the in-house prepared collagen. A mixture consisting of rBSP (50 µg/ml) and type I collagen (5 µg/ml) was incubated overnight at 4°C and rotary-shadowed with platinum. Analysis of multiple TEM copper grids shows the mean rBSP binding site on type I collagen to be 31% of the total distance from one end of the collagen molecule with a standard deviation of 5.58% (at the 95% confidence level) (Figure 3.6A). Subsequently, TEM studies of the rBSP-collagen interaction with the collagen obtained from Sigma was completed and based on the results of binding of both N-terminal collagen antibody and rBSP, it appears rBSP binds closer to the N-terminal end (Figure 3.6B). Furthermore, this suggests that BSP covers a region that is approximately 315±57 (at the 95% confidence level) residues from the N-terminal end of the collagen molecule, corresponding to a region encompassing residues 258 to 372. Figure 3.6B was not included in the determination of the mean and standard deviation as the length of the C-terminus was not well-defined and therefore a significant margin of error would result.

After analysis of all the TEM copper grids, it was evident the main difficulty was locating monomeric type I collagen molecules with both rBSP and anti-type I collagen antibody bound to it. In most instances, either unbound molecules (rBSP, anti-type I collagen antibodies and type I collagen) or singly bound proteins were observed.
Figure 3.3 Rotary-shadowing TEM of recombinant BSP.

rBSP (50 µg/ml) was visualized by (B) rotary-shadowing with platinum at an angle of 5° and a single BSP molecule was selected for magnification (D). The buffer (70% glycerol/30% 0.01 M ammonium formate) was rotary-shadowed and served as the control (A). The schematic drawing [adapted from (9)] (C) illustrates the shape of rBSP particle found in (B, D) and in the literature (9). Scale bar = 100 nm.
**Figure 3.4 Rotary-shadowing TEM of type I collagen molecules.**

Type I collagen (prepared in-house and from Sigma) was dissolved in 70% glycerol/30% 0.01M ammonium formate to a concentration of 5 µg/ml and rotary-shadowed. Both preparations of type I collagen have the characteristic semi-flexible, rod-like structure of monomeric type I collagen molecules (red rectangles). In addition, apparent type I collagen aggregates are observed with the in-house prepared collagen. The samples were analyzed in a Philips CM-10 TEM operating at 80 kV. Magnification 130,000 x. Scale bar = 100 nm
Figure 3.5 Rotary-shadowed N-terminal type I collagen antibodies with platinum. Rotary-shadowed antibodies to type I collagen (5µg/ml) possess a globular appearance with a diameter of approximately 10 nm. Representative anti-type I collagen antibodies are indicated by purple arrows. Magnification 130,000 x. Scale bar = 100 nm
Figure 3.6 Binding of rBSP on type I collagen.

rBSP (50 µg/ml) and type I collagen (prepared in-house; 5 µg/ml) were incubated overnight at 4°C and rotary-shadowed with platinum (A). The binding location of rBSP on type I collagen is indicated by the white arrows. In addition, TEM studies were completed with the collagen obtained from Sigma and the binding of the N-terminal collagen antibody (black arrow) and rBSP are indicated (white arrow) (B). The binding location of rBSP on type I collagen is at a position of approximately 130 nm (white arrow) from the N-terminal end of the collagen molecule. Measurements were made using the ImageJ software. The total length of the individual collagen molecules and the distance of the rBSP binding site measured from N-terminal end of the collagen molecules are indicated. Magnification 130,000 x. Scale bar = 100 nm
3.4 DISCUSSION

The aim of the present study was to gain insight into the BSP-type I collagen interaction. We have previously determined that the BSP-type I collagen interaction is mainly hydrophobic in nature, but stabilized by electrostatic forces. Additionally, the type I collagen-binding region has been determined to be residues 18-45 on BSP, a highly conserved region that is rich in hydrophobic and basic amino acids (11). However, the BSP-binding region on type I collagen is unknown. In this study, using rotary-shadowing TEM, the BSP-binding site was localized on type I collagen.

Rotary-shadowing TEM shows rBSP as a monomer with a globular structure that is linked to an elongated thread-like structure, which is consistent with the findings of Wuttke et al. Moreover, Wuttke et al., proposed the globular structure of rBSP, expressed from human embryonic kidney (HEK 293) cells, to be the C-terminal region since it does not contain any glycans. In contrast, the thread-like structure contains the highly glycosylated structure located at residues 180-220 (9). However, with regards to the prokaryotically-expressed rBSP used in our studies, the globular structure is likely due to the high levels of hydrophobic residues, while the thread-like structure is likely due to the high content of acidic amino acids (12).

Both types of collagen used in this study were extracted from rat tail tendons which have been shown to be a good source of monomeric collagen. However, a small content of covalently cross-linked polymeric collagen is likely to be present as cross-linking cannot be completely prevented or reversed (13). These cross-linked polymeric collagen molecules were observed with the in-house prepared collagen. With regards to the monomeric collagen molecules observed, the lengths of these molecules were
consistent with the findings of Dahl et al., which found the mean length to be 250 nm and standard deviation to be 48 (5). Unexpectedly, platinum-shadowed collagen molecules from Sigma were lighter in colour than the platinum-shadowed collagen molecules prepared in-house. This is likely due to a smaller amount of the contrast-enhancing metal (platinum) being evaporated onto the protein surface which is believed to have occurred during the processing of these samples in our studies (1-3).

The BSP-collagen interaction is only partly electrostatic and, in fact, it is mainly hydrophobic in nature (11). These findings parallel those of Kasugai et al., which determined that bone fragments demineralized with 4 M guanidine-HCl (a strong dissociative denaturant) still contained native BSP bound to type I collagen (14, 15). Therefore, it is postulated that BSP binds along the collagen molecule at a site with a surface chemistry that is, at least in part, hydrophobic. Type I collagen has an isoelectric point of 9.1 and a net charge of ~40+ at physiological pH. The molecule has a total of 270 basic residues (Arg, Lys, Hyl, and His) and 222 acidic residues (Asp and Glu). However, there are three regions along the collagen molecule that contain areas of high hydrophobicity. Two of the regions are in an overlap zone (c_2-c_1, which corresponds to residues 213-248) and (a_4-a_3, residues 307-324), while one of the regions is in a gap region (a_1-e_2, residues 342-374). Of relevance, Maitland et al., proposed that the hydrophobic domains of NCPs are involved in binding to the hydrophobic domains of type I collagen (16). Correlating these findings with the known triple-helical type I collagen sequences, the BSP-binding region (residues 255 to 372) comprises two regions along the collagen molecule that are high in hydrophobic amino acids, (a_4-a_3, a region encompassing residues 307-324) and (a_1-e_2, residues 342-374).
Using a far-Western blotting technique, Fujisawa et al., found that BSP bound to the $\alpha_2$ chain of the collagen molecule. However, there are many potential issues with this study including the non-specific binding to the $\alpha_1$ chain and that the collagen utilized in this study was not in a triple-helical form (18). Previous work demonstrated that the triple-helical structure of type I collagen is important for interacting with BSP and it is likely that at least two of the $\alpha$ chains are involved (19). San Antonio et al., mapped the binding sites of hundreds of type I collagen ligands onto a 2-D model of the fibril and the distribution of the binding sites suggest these can be organized into two domains: the “cell interaction domain” and the “matrix interaction domain”. The “cell interaction domain” comprising an overlap region contains integrin-mediated cell-to-cell interactions, while the “matrix interaction domain”, overlapping a gap region, contains ECM interactions.

We have previously demonstrated that two glutamic acid-rich regions are responsible for the nucleating activity, and this activity is increased 10-fold upon binding to collagen. Studies on calcified turkey tendon and in bone have shown initial mineral formation occurring in the gap regions of type I collagen (21-23). Moreover, Landis et al., proposed that the packing of consecutive 2-D collagen arrays into 3-D assemblages results in the formation of channels or gaps which are postulated to be the sites of nucleation. Furthermore, given the pore space between adjacent type I collagen molecules is 0.24 nm and the unstructured, flexible nature of BSP, it is postulated that the glutamic-acid regions can extend into the gap region of the adjacent collagen molecule and initiate mineral formation. Of relevance, DPP, a proposed mediator of mineralization
in dentin, has been shown to bind primarily to the fibril surface at the “e” band that is present in the middle of a gap region (24).

In conclusion, our findings suggest that the N-terminal hydrophobic sequence (residues 18-45) of BSP binds to collagen at a region that encompasses residues 258 to 372. This corresponds to BSP binding an overlap region of the collagen fibril by its N-terminal collagen-binding domain and the two glutamic acid rich regions binding in the gap region of the adjacent collagen molecule where it initiates mineral formation (Figure 3.8)
Figure 3.7 Schematic of the triple-helical type I collagen sequences of the BSP binding site.

The BSP-binding region on type I collagen is approximately 315±60 residues from the N-terminal end of the collagen molecule, or residues 258 to 372. (A) Residues 255 to 342 (purple rectangle) lie in an overlap region while (B) residues 343 to 372 (orange rectangle) lie in a gap region of the collagen fibril. The basic (Lys and Arg) and acidic residues (Asp and Glu) are shown in bold. Regions with a high concentration of charged residues are enclosed within rectangles and the letters correspond to the bands observed after positive staining of the collagen fibril. [Adapted from (17)]
**Figure 3.8 Model of BSP-mediated hydroxyapatite nucleation.**

(A) BSP binds to type I collagen at a site that is approximately 31% from the N-terminal end of the molecule. (B) This corresponds to BSP binding an overlap region of the collagen fibril by its N-terminal collagen-binding domain (purple) and the two glutamic acid rich regions (brown) binding in the gap region of the adjacent collagen molecule where it initiates mineral formation.
3.5 REFERENCES


CHAPTER FOUR
4.1 INTRODUCTION

The extracellular matrix (ECM) is a dynamic structure that constantly undergoes changes in response to stimuli such as inflammation, wound healing and cancer (1). There are many proteinases that mediate the degradation of the ECM including the matrix metalloproteinase (MMP) family, the adamalysin-related membrane proteinases (ADAMs) family and tissue serine proteinases such as thrombin (2).

Thrombin is a multifunctional protease that is important in a number of biological processes such as blood coagulation and bone formation. The precursor of thrombin, prothrombin, (also known as Factor II), is produced in the liver and secreted into the blood circulation. Upon injury, prothrombin is converted to thrombin by the prothrombinase complex for blood clot formation (3). The involvement of thrombin in bone repair is primarily via the activation of protease-activated receptor 1 (PAR-1), a G protein-coupled receptor expressed by osteoblasts, thereby mediating proliferation of these cells (4). Thrombin also inhibits apoptosis of osteoblasts (5). In addition, thrombin cleaves a number of ECM proteins such as osteopontin (OPN) (6) and bone sialoprotein (BSP) (7).

Previous studies from our laboratory have shown that thrombin cleaves rat BSP at Arg27-Tyr28, a site that is conserved in mouse, pig and human, but not in cow, which has a thrombin-cleavage site at Arg122-Lys123 (7). Interestingly, thrombin cleaves rat BSP in the middle of the collagen-binding domain (BSP18-45), which effectively abolishes collagen-binding (unpublished data). The physiological relevance of this conserved thrombin cleavage site, however, has not been fully elucidated and is the main focus of this study.
In addition to hydroxyapatite (HA) nucleation, acidic phosphoproteins of the ECM also function as signaling molecules in physiological pathways (8, 9). BSP contains a highly-conserved C-terminal RGD sequence that interacts with the \( \alpha_v \beta_3 \) integrin and has been shown to be important in cell adhesion and migration of osteoblast and tumor cells (8-12). In addition, BSP has been shown to promote angiogenesis by facilitating the migration and attachment of human umbilical vein endothelial cells (13).

Since BSP has been shown to be a chemotactic agent, we hypothesize that, upon bone injury, thrombin becomes activated, then cleaves and releases BSP into the ECM. Subsequently, BSP can act as a signal molecule to recruit osteoprogenitor cells that will differentiate into osteoblasts. The purpose of this study was to determine whether thrombin cleaves and releases BSP from type I collagen. Thrombin-digestion reactions were completed and analyzed using SDS-PAGE and staining with Stains-All and silver, Western blotting and MALDI-MS.

### 4.2 MATERIALS AND METHODS

#### 4.2.1 MATERIALS

Human alpha-thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Type I collagen was extracted from rat tail tendons in our laboratory as previously described (14). Sprague-Dawley rats (250-300 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Amino-caproic acid, benzamidine HCl, \( N \)-ethyl maleimide and phenylmethylsulfonyl fluoride (PMSF) and \( N \)-Tris(hydroxymethyl)methyl-2 aminoethanesulfonic acid (TES) were all purchased from Sigma-Aldrich (St. Louis, MO).

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ammonium persulfate (APS), \( N, N, N', N' \)-tetramethylethane-1,2-diamine (TEMED),
acrylamide/bis-acrylamide, SDS and pre-stained protein standards were purchased from Bio-Rad Laboratories (Hercules, CA). β-mercaptoethanol and glycine were obtained from Thermo Fisher Scientific (Rockford, IL). Isopropanol was from VWR International (West Chester, PA). Imidazole, Stains-All and silver nitrate were purchased from Sigma-Aldrich. Anti-BSP was purchased from abcam® (Toronto, Ontario). Hybond-P polyvinylidene fluoride transfer (PVDF) membranes, enhanced chemiluminescence (ECL) reagents, hyperfilm-ECL film and horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were obtained from GE Healthcare (Amersham).

4.2.2 METHODS

4.2.2.1 Thrombin cleavage of recombinant BSP (rBSP) bound to type I collagen

An overview of the thrombin-digestion experiments on rBSP bound to type I collagen is illustrated in Figure 4.1. rBSP (10 µg) was incubated with either (1) type I collagen fibrils or (2) soluble type I collagen that was subsequently allowed to form fibrils. The two set-ups are described below.

a) **Incubation of rBSP after collagen fibril formation**

Type I collagen (4 mg/ml) fibril formation was induced with the addition of 250 µl TES-phosphate buffer (270 mM NaCl, 30 mM Na₂HPO₄ and 60 mM TES pH 7.5) at 37°C and incubated for 4 h. Subsequently, rBSP (10 µg) was added to the collagen fibrils and incubated overnight at 4°C. This should result in rBSP binding to the surface of the collagen fibrils.

b) **Incubation of rBSP before collagen fibril formation**

rBSP (10 µg) was incubated with soluble type I collagen (4 mg/ml) overnight at 4°C. Subsequently, fibril formation was induced with the addition of TES-phosphate
buffer at 37°C for 4 h. This should result in some of the rBSP being internalized within
the collagen fibrils.

In both set-ups (a and b), unbound rBSP was first removed by buffer exchange
using Amicon® Ultra-0.5 centrifugal filter units. A total of five buffer exchanges were
performed. The BSP-collagen complexes were then incubated with thrombin (0.08 U) in
digestion buffer (50 mM Tris HCl, 10 mM CaCl₂, and 1X PBS) at 22°C for 2 h. After
recovery of supernatant, the two different preparations of rBSP and type I collagen were
then incubated with 10% SDS at 22°C for 1 h to release any rBSP that was still
associated with the collagen fibrils. The recovered solutions were analyzed by 12% SDS-
PAGE gel and staining with Stains-All and silver as previously described (15) or loaded
onto a 6% SDS-PAGE gel and transferred onto a PVDF for western blot analysis. Briefly,
membranes were blocked with 5% milk powder in phosphate-buffered saline containing
Tween-20 (PBS-T) and incubated overnight at 4°C. The membranes were then incubated
with anti-BSP (1:500 dilution) for 1 h followed by incubation with anti-rabbit-HRP
antibodies (1:1000 dilution, GE Healthcare) for another hour. Four consecutive washes
(10 min) with PBS-T were completed between incubation steps. The membranes were
then developed with ECL reagents (5 min) and analyzed using the ChemiImager 5500 gel
doc system and corresponding software (Alpha Innotech).

4.2.2.2 Thrombin cleavage of native BSP (nBSP) and demineralized rat bone
fragments

Rat bone nBSP was extracted as previously described (16). nBSP (5 µg) was
incubated with thrombin (0.08 U) in thrombin-digestion buffer at 22°C for 2 h. The
reaction solutions were then concentrated and loaded onto a 12% SDS-PAGE gel and stained with Stains-All and silver for analysis (15).

To prepare demineralized bone chips for study of thrombin susceptibility, an established bone extraction protocol was followed (16). Briefly, adult rat tibia, femur, humerus, radius and ulna were excised and fragmented into large chunks with a hammer and chisel. Subsequently, the bone fragments were further broken down with a mortar and pestle under liquid nitrogen. The bone chips were then freeze-thawed with 1X PBS containing proteinases inhibitor (PI; 100 mM amino-caproic acid, 5 mM benzamidine HCl, 5 mM N-ethyl maleimide and 1 mM PMSF). This was repeated for a total of four times. Bone chips were then incubated with chloroform: methanol (3:1) at 4°C for 2 h to remove adipose tissue. 1X PBS (containing PI) was then used to remove the chloroform: methanol. Subsequently, noncollagenous proteins were extracted from the chips using 4 M GuHCl/50 mM Tris buffer, pH 7.4, containing PI (1 l a day per 100 g bone) for four days. A 24-h wash with 1X PBS (exchanged three times) was then used to remove the 4 M GuHCl/Tris buffer. Following this, mineral-associated proteins were extracted using 0.5 M EDTA/ 50 mM Tris buffer, pH 7.4, containing PI (50 ml of EDTA solution per gram of bone per day) for four days. After the removal of the EDTA extract solutions, bone fragments were washed with 1X PBS for a total of three times.

The bone fragments (known to contain BSP bound to type I collagen) (17, 18) were then incubated with various concentrations of thrombin (0.02 – 0.08 U) in thrombin-digestion buffer at 22°C for 2 h. The reaction solutions were concentrated to 40 µl and loaded onto a 6% SDS-PAGE gel and transferred onto a PVDF membrane for
western blot analysis as described earlier or loaded onto a 12% SDS-PAGE gel and stained with Stains-All and silver (15).

4.2.2.3 Matrix-assisted laser desorption mass spectrometry (MALDI-MS) analysis

The collagenous residue of the demineralized rat bone fragments (after incubation with thrombin) and non-digested nBSP were analyzed by MALDI-MS (Bruker® Reflex IV, MALDI TOF MS, Department of Biochemistry, UWO). Sample aliquots of 2 µl were mixed with 2 µl of sinapinic acid in 50 % methanol/0.5% trifluoroacetic acid and spotted onto a 96-well MALDI plate. The plate was analyzed using a Reflex IV MALDI-TOF instrument in positive-ion mode. Mass spectra were collected and peaks were assigned using the XTOF software (Bruker, Germany).
Figure 4.1 An overview of the thrombin digestion experiments of rBSP bound to type I collagen. rBSP (10 µg) was incubated with either type I collagen fibrils (left) or soluble type I collagen (right) that was subsequently allowed to form fibrils. Prior to the addition and incubation of thrombin, unbound rBSP was removed by buffer exchange. The thrombin-digestion products were analyzed by Stains-All and silver.
4.3 RESULTS

4.3.1 Thrombin cleavage of rBSP bound to type I collagen

The two different preparations of rBSP and type I collagen were incubated with thrombin and the products were analyzed by Stains-All and silver staining and western blot. To ensure that the rBSP detected was the result of thrombin cleavage and not unbound rBSP, multiple washes were done using 1X PBS (prior to the incubation with thrombin). The first and fifth washes were also loaded onto the gels (Figures 4.2A and 4.2B, W1 and W5).

A band of approximately 55 kDa is apparent in the first washes and after addition and incubation with thrombin (Figures 4.2A and 4.2B, W1 and +thrombin lanes) from both rBSP-collagen preparations. However, the intensity of the band in the +thrombin lanes at 55 kDa from the rBSP incubated with fibrillar collagen is stronger than the corresponding band recovered from the preparation of rBSP preincubated with monomeric collagen prior to reconstitution (Figure 4.2A[a]). Following incubation with thrombin, both rBSP-collagen preparations were incubated with 10% SDS to recover all non-covalently bound protein. A band of approximately 55 kDa is apparent (Figure 4.2A, +SDS lanes). This suggests that the rBSP detected after incubation with thrombin was released from the surface of the (a) collagen fibrils or (b) within the collagen fibrils. However, the intensity of the bands in (Figure 4.2B, rBSP only and + thrombin lanes) are not expected based on the intensity of the bands in (Figure 4.2A, rBSP only and + thrombin lanes), a possible explanation for this is that full-length rBSP reacts poorly with anti-BSP.
4.3.2 Analysis of nBSP and demineralized rat bone fragments for thrombin susceptibility

nBSP was analyzed for thrombin susceptibility. Non-digested nBSP has an apparent Mr of 80 kDa, whereas after incubation with thrombin a second band at approximately 60 kDa is apparent (Figure 4.3A, +thrombin).

Rat bone fragments that were first demineralized were also analyzed for thrombin susceptibility and release of BSP. The collagenous residue after EDTA was incubated with various concentrations of thrombin (0.02 – 0.08 U) in thrombin-digestion buffer at 22°C for 2 h and the products were analyzed by SDS-PAGE and stained with Stains-All and silver and western blotting. From the western blot analysis, solutions recovered from incubated bone chips without thrombin contained BSP-immunoreactive bands with apparent molecular weights of 180, 80 and 60 kDa (Figure 4.3C, -thrombin lane). Addition of increasing amounts of thrombin resulted in a corresponding increased recovery of bands at 180, 80 and 60 kDa and evidence of lower-molecular-weight peptides (Figure 4.3C, +thrombin lanes). It is believed that the slower-migrating protein (180 kDa) represents polymers of BSP. Stains-All and silver staining of the recovered proteins after thrombin incubation shows a mixture of proteins with a prominent band at approximately 60 kDa (Figure 4.3B, +thrombin lane).

4.3.3 Matrix-assisted laser desorption mass spectrometry (MALDI-MS) analysis

The thrombin-digested reactions of the demineralized rat bone fragments and controls containing no thrombin were analyzed by MALDI-MS. The non-digested rat native BSP (control) gave rise to a peak at 52,423 m/z (Figure 4.4A) whereas the thrombin-digested rat native BSP gave rise to a peak at approximately 47,500 m/z
resulting in a loss of approximately 5000 m/z. Thrombin gave rise to a peak at 36,708 m/z. MALDI-MS analysis of the thrombin-digested demineralized bone fragments reveals a major peak at 43,784 m/z and minor peaks at 34,112 m/z, 28,069 m/z, 22,025 m/z and 13,727 m/z (Figure 4.4D). This is not consistent with the western blot analysis (Figure 4.3A), suggesting that non-BSP proteins in the sample were identified by MS.
Figure 4.2 Analysis of thrombin-digestion reactions of rBSP bound to type I collagen.

Prior to the addition and incubation with thrombin, unbound rBSP from the two different rBSP-collagen preparations - (a) rBSP on the surface of collagen fibrils and (b) some rBSP internalized within the collagen fibrils - was removed with a total of five buffer exchanges using 1X PBS. Following this, (a) and (b) were incubated with 10% SDS. The products (the first and fifth washes, incubation with thrombin and 10% SDS) were collected and loaded onto a (A) 12% SDS-PAGE gel and stained with Stains-All and silver for analysis or (B) loaded onto a 6% SDS-PAGE gel and transferred onto a PVDF membrane and incubated with anti-BSP antibodies to probe for BSP. A band corresponding to the release of rBSP from type I collagen fibrils is apparent at 55 kDa [W1, +thrombin and +SDS lanes in (a) and (b)]. “M” is the molecular marker.
Figure 4.3 Analysis of nBSP and demineralized rat bone fragments for thrombin susceptibility.

nBSP was incubated with thrombin and the reactions were concentrated and loaded onto a (A) 12% SDS-PAGE gel and stained with Stains-All and silver for analysis. Additionally, rat bone fragments that were first demineralized with EDTA was incubated with various concentrations of thrombin in digestion buffer at 22°C for 2 h. The reactions were loaded onto a (B) 12% SDS-PAGE gel and stained with Stains-all and silver for analysis or loaded onto a (C) 6% SDS-PAGE gel and transferred onto a PVDF membrane and incubated with anti-BSP antibodies to probe for BSP. Bands corresponding to the release of nBSP (C; red box) were present when incubated at all concentrations of thrombin. “M” is the molecular marker.
Figure 4.4 MS analysis of thrombin-digested demineralized bone chips

(A) Mass spectrum of non-digested rat BSP with a dominant peak at 52,423 m/z.

(B) Mass spectrum of thrombin-digested rat BSP with a dominant peak at approximately 47,500 m/z.

(C) Mass spectrum of thrombin with a dominant peak at 36,708 m/z.

(D) Mass spectrum of thrombin-digested rat bone showing a dominant peak at 43,784 m/z and minor peaks at 34,112 m/z, 28,069 m/z, 22,025 m/z and 13,727 m/z.
4.4 DISCUSSION

Bone formation and remodeling are complex, yet still poorly understood, processes that involve many factors. The acidic phosphoproteins of the ECM have been shown to function as signaling molecules in physiological pathways. The aim of the present study was to gain insight into the conserved thrombin-cleavage site of BSP. Previous studies from our laboratory have shown that thrombin cleaves rat BSP at Arg27-Tyr28. In this study, we present evidence that rBSP bound to type I collagen and demineralized rat bone fragments (containing nBSP bound to type I collagen) are susceptible to thrombin cleavage. This was confirmed with Stains-All and silver, western blot and MALDI-MS analysis.

nBSP was analyzed for thrombin susceptibility. A 60-kDa band was apparent after incubation with thrombin. This was not expected based on the thrombin-cleavage site at Arg27-Tyr28. Previous unpublished data from our laboratory show non-digested rBSP with a peak at ~34,754 (m/z), while the thrombin-digested rBSP had a peak at ~30,761 (m/z), resulting in a loss of approximately 4,000 m/z. Therefore, the expected BSP fragment would be approximately 4 kDa less than the full-length and this loss may not be evident on SDS-PAGE. This shortened BSP fragment at 60 kDa was also evident in the western blot analysis of demineralized rat bone fragments which is likely due to the slow migration of BSP. Moreover, the additional bands below 60 kDa are possibly proteolytic fragments of BSP while the high-molecular-weight bands may be a result of transglutaminase, a protein-crosslinking enzyme that generates polymers of BSP (19). This observation of a shortened BSP fragment at 60 kDa is consistent with the findings of Gorski et al., where proteolytic fragments of BSP (45-50 kDa) were found in bone.
mineralization foci (BMF). Moreover, in the presence of a serine protease inhibitor 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), cleavage of BSP was inhibited. This suggests the BSP fragments were a result of serine protease activity, which thrombin is a serine protease (20).

The MS peak at 52,423 m/z for the non-digested BSP (control) coincides with the findings of Zaia et al. (21) and Wuttke et al. (22). However, the dominant MS peak at 43,784 m/z for thrombin-digested bone was not expected based on the thrombin-cleavage site at Arg27-Tyr28. The expected peak for the demineralized rat bone fragments (after incubation with thrombin) would be closer to 47,500 m/z based on the thrombin-digested BSP. Since the Stains-All and silver staining of the demineralized rat bone fragments showed a mixture of proteins, this suggests the unexpected MS peak is not likely BSP. Although MS has been demonstrated to be a good tool in proteomic analysis, the highly acidic nature of BSP appears to be a problem for detection by MS. Previous studies from our laboratory have shown that a chondroitin sulfate proteoglycan, with a highly acidic protein core, was not detectable by MS (unpublished data). A potential explanation for this is that the less-negatively charged proteins in the sample mixture are more easily protonated and detected by MS than BSP.

For proteins to become functional, often proteolysis is required. Also, processing of protein through proteolysis could potentially modulate its functional roles. Many polypeptides isolated from bone and dentin have been shown to be distinct proteolytic-derived large peptides. In some instances, it has been demonstrated that these cleavages are necessary for protein function. Dentin sialophosphoprotein (DSPP) the inactive precursor, is cleaved by PHEX, resulting in two active fragments, dentin sialoprotein
(DSP) and dentin phosphophoryn (DPP) (23). Similarly, dentin matrix protein 1 (DMP-1) exists as 37-kDa and 57-kDa peptides and it has been proposed that this cleavage is important in osteogenesis and dentinogenesis (24). Of relevance, thrombin also cleaves OPN at a Gly-Arg-Gly-Asp-Ser (GRGDS) site into an N-terminal and a C-terminal fragment. Many studies have analyzed the physiological relevance of this cleavage. Grassinger et al., demonstrated that the N-terminal thrombin-cleaved OPN fragment acts as a chemotactic agent for hemopoietic stem and progenitor cells via α9β1 and α4β1 integrins (25). Similarly, thrombin-cleaved OPN fragments have been shown to induce migration of a variety of cell types including monocytes, macrophages, neutrophils and lymphocytes (26-29).

There are several physiological processes that require the migration of osteoprogenitor cells. During the normal processes of bone remodeling and bone repair, there is a recruitment of osteoprogenitor cells responsible for generating mature bone. Although there are numerous factors within bone that can stimulate cell migration such as cytokines, the restricted expression of BSP to cells of osteogenic origin suggests BSP may act as an ECM signal. Moreover, BSP has been shown to act as a chemotactic agent for osteoblast and cancer cells through the activation of the focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) signaling pathway (8-12, 30). FAK is a tyrosine kinase that is activated when it is phosphorylated at Tyr397 (31). Following, FAK associates with various SH2/SH3 domain-containing proteins including Src and Grb2 (32). This results in the activation of the mitogen-activated protein kinase (MAPK) pathway, such as ERK via the GTPase such as Ras (33). ERK has been demonstrated to be important in cell migration through the activation of downstream targets such as
members of the activator protein-1 (AP-1) (34). BSP has been demonstrated to influence osteoblast differentiation (10) through the activation of osteoblast-related transcription factors including Runx2 (35), alkaline phosphatase and Osx (36).

In the present study, we demonstrated that BSP bound to type I collagen is susceptible to thrombin cleavage. We speculate that BSP released from bone by thrombin may function as a chemotactic agent in bone repair. In order to evaluate this hypothesis, we will have to determine whether intact and/or degraded BSP is released from the mineralized collagenous matrix after injury and whether thrombin-cleaved BSP from collagen can mediate osteoblast differentiation. The results from these studies will provide further insight into the physiological significance of this cleavage.
4.5 REFERENCES


CHAPTER FIVE
5.1 SUMMARY

Biomineralization is a complex, yet poorly understood, process that involves many factors. The precise mechanisms of crystal nucleation and growth are unknown. The highly organized arrangement of hydroxyapatite (HA) within the collagen matrix suggests that mineralization in bone is a tightly regulated process. Furthermore, dysregulation results in pathological conditions such as osteogenesis imperfecta. In normal calcified bone, the HA crystals tend to be plate-like (with average dimensions of ~45 nm in length, ~25 nm in width and 3-6 nm thick) and are aligned parallel to one another and with their long axes parallel to the collagen fibrils (1, 2). In contrast, the HA crystals in pathologically calcified tissues, may be thick, bulky blocks or narrow spear-shaped structures with dimensions that are three-to ten-fold larger than normal HA crystals (3), or may be similar in shape (plate-like) to normal crystals, but thinner (4). Additionally, the abnormal HA crystals are not aligned with respect to the long axis of the collagen fibrils (3, 4). Irregularities in the collagen matrix, where the overlap and gap regions are mismatched, is proposed to be responsible for the various sizes and shapes of HA crystals found in pathological calcified tissues.

While there are several postulated mechanism for biomineralization, one prominent mechanism requires preexistence of a surface such as type I collagen for ion deposition and nucleation by acidic proteins (5-7). The collagen matrix neither promotes nor inhibits HA nucleation, however; an acidic phosphoprotein of the ECM is proposed to be play this role. Bone sialoprotein (BSP) is a likely candidate due to its spatio-temporal pattern of expression and HA-nucleating capabilities (8-10). Moreover, since this nucleation activity is increased upon binding to collagen, it is postulated that BSP is
secreted into the ECM by osteoblasts, binds to type I collagen in the gap regions (where initial mineral formation is seen to occur), or the regions adjacent to it, and mediates mineral formation.

Previous work demonstrated that the BSP-collagen interaction is mainly hydrophobic in nature, but stabilized by electrostatic forces. The collagen-binding region has been located to a region spanning residues 18-45 on BSP, a highly conserved region that is rich in hydrophobic and basic amino acids (11). The BSP-collagen interaction requires a native triple-helical type I collagen structure, but does not require either collagen telopeptides or fibrils (12). Furthermore, post-translational modifications (PTMs) are not involved in collagen-binding since both rat-bone-extracted BSP and recombinant BSP (rBSP; unmodified BSP) demonstrate comparable binding affinities. Although much research has been devoted to studying the BSP-collagen interaction, the BSP-binding region on type I collagen has not been identified and was the main focus of this thesis.

In chapter two, a chemical cross-linking method was initially used to determine the BSP-interacting domain on type I collagen. These experiments involved the incubation and conjugation of single-cysteine rBSP (1-100) mutants to the sulphydryl reactive, photoactivable cross-linkers APB and APDP. Type I collagen was then added and irradiated with UV light to induce cross-link formation. Multiple approaches and modifications were taken in an effort to improve the efficiency and/or recovery of BSP-collagen cross-linked products. However, the yield of the cross-linked products was low in all instances and therefore the complexes were not further characterized. It is still a puzzle as to why the chemical cross-linkers did not result in sufficient cross-linked
products, as both cross-linkers have been used successfully in many cross-linking experiments studying protein-protein interactions. A possible explanation lies in the cross-linkers’ spacer arms; perhaps these were not long enough to reach a collagen amino acid from the cysteine residue of the single-cysteine rBSP (1-100) peptide. Unfortunately, these two cross-linkers possess the longest spacer arms among commercially available heterobifunctional cysteine-reactive cross-linkers with a photoactivable group. Another potential explanation is that the current method of detection was not sufficiently sensitive to detect the BSP-collagen cross-linked products. Therefore, due to these difficulties in obtaining sufficient BSP-collagen cross-linked products, an alternative protocol using rotary-shadowing TEM was used.

Rotary-shadowing TEM has been utilized extensively for identifying the binding site(s) of various proteins on collagen as it offers high-contrast visualization of individual macromolecules. In chapter three, this method was used to visualize the BSP-interacting domain on collagen. This involved rotary-shadowing with platinum full-length prokaryotically expressed rBSP, type I collagen (extracted from rat tail tendons) and an anti-type I collagen antibody recognizing the N-terminal end of collagen molecules. Rotary-shadowing TEM shows rBSP as a monomer possessing a globular structure of ~10 nm linked to an elongated thread-like structure of ~25 nm, while type I collagen appeared as semi-flexible molecules. These structures are consistent with the findings in the literature (13, 14). The locus of interaction is approximately 31% from the N-terminus based on studies in which BSP, type I collagen and an antibody recognizing the N-terminal end of the collagen molecule were combined. Taking this into consideration, as well as the arrangement of the collagen fibril, the highly flexible structure of BSP and the
hydrophobic nature of the BSP-collagen interaction, I postulate that the N-terminal hydrophobic sequence (residues 18-45) of BSP binds collagen in an overlap region at a site that is rich in hydrophobic residues, while one or both of the glutamic acid-rich regions extend into the gap region of the adjacent collagen molecule.

In addition to HA nucleation, BSP has been demonstrated to function as a signaling molecule. BSP contains a highly conserved C-terminal RGD site that interacts with the αvβ3 integrin and has been shown to be important in osteoprogenitor cell migration and differentiation (15-17). Within the collagen-binding domain of BSP, there is a highly conserved thrombin cleavage site at Arg27-Tyr28. Thrombin cleaves rat BSP in the middle of the collagen-binding domain (residues 18-45), which effectively abolishes collagen binding, implying physiological significance. To address the relevance of this, analysis of the products of the incubation of thrombin with rBSP bound to fibrillar collagen and with demineralized bone chips was completed. Analysis with SDS-PAGE and western blotting demonstrated that rBSP bound to type I collagen fibrils is susceptible to thrombin cleavage allowing for its release from the collagen complex. Similarly, endogenous BSP present in demineralized bone chips is also released from these chips with thrombin incubation. Since BSP has been shown to function as a chemotactic agent, I hypothesize that, upon bone injury, thrombin becomes activated, cleaves and releases BSP into the ECM. Subsequently, BSP can act as a signal molecule to recruit osteoprogenitor cells which will differentiate into osteoblasts.

**Conclusions and Future Directions**

The first objective of this thesis was to ultimately map the BSP-binding region on type I collagen down to the amino-acid level. As the present studies provide qualitative
insight into the BSP-binding domain on collagen, future studies will be important to fully elucidate this domain and to determine how this interaction promotes nucleation of HA. Future TEM studies to validate the BSP-binding domain on collagen could involve the use of the biotinylated rBSP (18-45) peptide present in the laboratory. This approach is promising since a shorter peptide than the rBSP (1-100) peptide may reduce the inherent variability noted in the site of interaction with the larger peptide. Furthermore, because of the strong binding affinity of avidin to biotin and the relatively large mass of avidin, the site of interaction should be readily apparent. In addition, synthetic triple-helical type I collagen peptides can be synthesized and utilized in solid-binding assays to further characterize the BSP-binding region. Researchers have extensively used synthetic triple-helical peptides of type II and III collagen to map protein- or peptide-binding locations (18). Moreover, the BSP-binding site on collagen determined in this present study can be used as constraints in molecular-modeling techniques to generate a predicted structure (19, 20). Additionally, structural studies such as $^1$H NMR, CD and X-ray scattering may be used to determine change in BSP conformation, if any, upon binding to collagen (21).

The findings of the second objective suggest that the release of BSP from bone may provide signals to initiate the bone repair process. In order to evaluate this hypothesis, it is important to determine whether full-length and/or degraded BSP is released from the mineralized collagenous matrix after injury and whether thrombin-cleaved BSP from collagen can mediate osteoblast differentiation. Although MS has been demonstrated to be a good tool in proteomic analysis, the highly acidic nature of BSP may pose a problem. In the present study, the thrombin-digested BSP in the demineralized bone mixtrue was not detectable by MS. A potential explanation is that in
a mixture of proteins, the less-acidic proteins are more easily protonated and detected by MS. Thus, additional purification and concentration of the acidic peptides prior to analysis by MS should be included, similar to what was done for the analysis of the highly phosphorylated osteopontin (22). The results from these studies will provide further insight into the physiological significance of this cleavage.

In cases where the injury is so severe that natural healing cannot take place, the use of biomaterials may aid the healing process. These materials can be classified into three groups (1) osteoinductive, (2) osteoconductive, and (3) calcigenic materials (23). Established osteoinductive materials, such as BMP, are involved in the recruitment and differentiation of osteoprogenitor cells into osteoblasts (24). However, disadvantages of BMP are the cost and the potential for serious side effects. While BSP has apparent osteoinductive properties in vitro, studies in vivo are not yet conclusive. Osteoconductive materials such as HA composites provide a scaffold upon which bone can form. However, these materials can result in disorganized mineralization of bone tissues (24). Finally, calcigenic materials are involved in inducing local calcification rather than bone formation, which would allow the bone defect itself to be stabilized and potentially repaired more rapidly. This involves a calcigenic agent binding to a collagen scaffold in order to produce a mineralized matrix that is biologically and structurally comparable to bone (25). A postulated calcigenic agent is BSP.

In conclusion, the findings from these studies have added novel information to the BSP-collagen interaction and its role in bone formation and mineralization. This may ultimately lead to the generation of therapeutic agents to promote bone repair.
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