**Structural Characterization of Human Recombinant and Bone-derived Bone Sialoprotein**

**FUNCTIONAL IMPLICATIONS FOR CELL ATTACHMENT AND HYDROXYAPATITE BINDING**

Martina Wuttke, Stefan Müller, D. Patric Nitsche, Mats Paulsson‡, Franz-Georg Hanisch, and Patrik Maurer

*From the Institute for Biochemistry II, Medical Faculty, University of Cologne, D-50931 Cologne, Germany*

Human bone sialoprotein (BSP) comprises 15% of the total noncollagenous proteins in bone and is thought to be involved in bone mineralization and remodeling. Recent data suggest a role for BSP in breast cancer and the development of bone metastases. We have produced full-length recombinant BSP in a human cell line and purified the protein from human bone retaining the native structure with proper folding and post-translational modifications. Mass spectrometry of bone-derived BSP revealed an average mass of 49 kDa and for recombinant BSP 57 kDa. The post-translational modifications contribute 30–40%. Carbohydrate analysis revealed 10 different complex-type N-glycans on both proteins and eight different O-glycans on recombinant BSP, four of those were found on bone-derived BSP. We could identify eight threonines modified by O-glycans, leaving the C terminus of the protein free of glycans. The recombinant protein showed similar secondary structures as bone-derived BSP. BSP was visualized in electron microscopy as a globule linked to a thread-like structure. The affinity for hydroxyapatite was higher for bone-derived BSP than for recombinant BSP. Cell adhesion assays showed that the binding of BSP to cells can be reversibly diminished by denaturation.

Bone sialoprotein (BSP) is a noncollagenous protein that was first isolated from bovine bone (1). The protein is very abundant as it comprises about 15% of the total noncollagenous proteins in bone. After cleavage of the signal peptide sequence the polypeptide chain of human BSP contains 301 amino acids. According to the cDNA sequence the molecular mass of the core protein is 33.6 kDa (2). However, after separation on SDS-PAGE the protein could be identified as an 80-kDa band (3, 4). The reason for these different sizes is most likely the high content of post-translational modifications, where carbohydrates were shown to contribute about 50% to the mass of the protein (5). However, the nature of the carbohydrates on human BSP has not been described. Additionally the protein was shown to be sulfated and phosphorylated (6, 7). Close to the C terminus the molecule contains an RGD cell-binding motif that enables the protein to bind integrin $\alpha_v\beta_3$ (8). There are three tyrosine-rich regions and two clusters rich in glutamic acids (9).

Bone sialoprotein is expressed by most cell types in bone, cartilage, and teeth. In the mineralized bone matrix the highest BSP concentrations were found in areas where bone is newly synthesized or remodeled (10, 11). Outside of the skeleton the protein was found in placental trophoblasts (12) and in platelets (13). Additionally, BSP is expressed in certain carcinomas that predominantly metastasize to bone, i.e. breast, prostate, lung, thyroid tumors, as well as multiple myeloma and neuroblastoma. Bone metastases of breast and prostate cancer patients expressed significantly more BSP than visceral metastases (14).

The function of BSP in bone and cartilage is still not fully understood. Using the RGD sequence the protein can bind cell surfaces of different mammalian cells. Therefore it could mediate the attachment of osteoblasts (15) and osteoclasts (16) to the bone matrix during bone remodeling. *In vitro* BSP was shown to promote bone resorption in a concentration-dependent manner (17) and thus the protein could be involved in bone degradation. As BSP expression is increased by glucocorticoids (18), hormones that support the differentiation of osteoblasts, it was postulated that the molecule is involved in the anabolic phase of bone remodeling (19). Additionally, the protein has a very high affinity for hydroxyapatite (20) and was shown to function as a de novo nucleator of hydroxyapatite crystals *in vitro* (21). For that reason BSP could be involved in the mineralization process of the bone, a functional role that is supported by the expression of BSP either right before or at the same time as when mineralization starts (22, 23). It is still unclear whether BSP is involved both in bone formation and in bone resorption or if only one of these functions is relevant *in vivo*. BSP-deficient mice showed some skeletal and tooth abnormalities at an age of 6 weeks (24). A detailed characterization of the animals has not yet been published.

Using immunoassay techniques elevated levels of BSP were detected in patients with certain metabolic and malignant bone diseases and BSP can be used as a diagnostic marker for bone remodeling (25). Additionally, the protein has clinical relevance as a prognostic serum marker for bone metastases. Primary breast cancer patients with preoperatively elevated serum BSP levels are at high risk of subsequent bone metastases in the first years after primary surgery. (26). Although the function of BSP in this context is still not clear, one hypothesis is that it is involved in the preferred attachment and metastasis of breast cancer cells in bone.
Most of the published investigations are based either on BSP that was isolated from bone under denaturing conditions or BSP fragments obtained from bacterial expression systems, accordingly not carrying post-translational modifications. Therefore, the aim of the present investigation was a comparative characterization of BSP expressed recombinantly in a human cell line with BSP isolated and purified under non-denaturing conditions from human bone with regard to folding and cell type specific post-translational modifications. Additionally, the influence of the molecular structure on functional properties of BSP was investigated.

**EXPERIMENTAL PROCEDURES**

**Recombinant Expression of BSP**—The cDNA clone for human BSP (plasmid B6-5g) was obtained from Dr. L. Fisher, NIDR, National Institutes of Health, Bethesda, MD (2). Using B6-5g as a template, the BSP cDNA sequence was amplified without its signal peptide from nucleotides 72 to 1025 under standard polymerase chain reaction conditions and cloned into an N-terminal His$_x$-Myc-enterokinase-tagged eukaryotic expression vector based on pCEP-Pu (27). The His$_x$-Myc sequence, and enterokinase sequences were either amplified by polymerase chain reaction or synthesized as single stranded oligonucleotides that were to be capable stranded DNA flow in the BM-40 signal peptide of the pCEP-Pu vector. The correct reading frame was confirmed by sequencing both strands using the ABI Prism 377 Sequencer with the Taq FS Dyex-deoxyterminator cycle sequencing kit (PerkinElmer Life Sciences/Applied Biosystems). Human embryonic kidney cells (EBNA-293, Invitrogen) were transfected with BSP expression plasmid DNA using the Fugene transfection reagent (Roche Molecular Biochemicals). Human embryonic kidney cells (EBNA-293, Invitrogen) were transfected with BSP expression plasmid DNA using the Fugene transfection reagent (Roche Molecular Biochemicals). Transfected cells were selected with 0.5 μg/ml puromycin in Dulbecco’s modified Eagle’s medium/F-12 medium following 3 days with 0.5M EDTA, pH 7.5. The EDTA extracts were electrophoretically transferred onto nitrocellulose following standard protocols (30). Following separation by SDS-PAGE, proteins were electrotheroferetically transferred onto nitrocellulose following standard protocols (30). After blocking with a wash with 4% Gdn-HCl in 50 mM Tris-HCl, pH 8.0, and stored at ~20°C.

**Purification of BSP from Human Bone**—Bone powder (300 g) was produced from femoral heads of patients undergoing hip surgery. Blood residues were removed by a wash with 4% Gdn-HCl in 50 mM Tris-HCl, pH 7.5. The Gdn-HCl was then removed from the bone powder by extensive washing. The noncollagenous proteins were extracted three times for 2–3 days with 0.5 μM EDTA, pH 7.5. The EDTA extracts were dialyzed against 50 mM Tris-HCl, pH 8.0, and loaded on a DEAE- Sepharose column equilibrated in 50 mM Tris-HCl, pH 8.5, and eluted with a linear sodium chloride gradient of 0–0.5 M NaCl followed by a second gradient from 0.5 to 1 M NaCl. All buffers were supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM benzamidine-HCl, and 1 mM 6-aminocaproic acid. BSP, eluted between 0.35 M and 0.85 M NaCl, was dialyzed against 50 mM Tris-HCl, pH 8.5, concentrated, and further purified using the strong anion exchange column ResourceQ (Amersham Pharmacia Biotech) and the same buffer. Finally, the protein was purified to homogeneity by using reversed-phase HPLC as described (28), where BSP was eluted with 31% acetonitrile, 0.05% heptfluorobutyric acid. Conformational changes of the protein at the low pH in the elution buffer were reversible after dialysis into 5 mM Tris-HCl, pH 8.0, as shown by CD spectroscopy. Homogeneity of the proteins was shown by SDS-PAGE (29) under reducing conditions and staining with silver nitrate.

**Western Blotting**—Following separation by SDS-PAGE, proteins were electrotheroferetically transferred onto nitrocellulose following standard protocols (30). After blocking with a 5% non-fat powdered milk solution, primary antibody (mouse anti-human BSP 1.2, monoclonal, Immunodiagnostics) was added (dilution 1:1000) for 1 h. The nitrocellulose was washed in TBS with 0.01% Tween 20 followed by a 1-h incubation with the secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse IgG, 1:2000, DAKO) in TBS with 5% non-fat powdered milk. Enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) were employed for signal detection with x-ray film according to the manufacturers protocol.

**Electron Microscopy**—To exclude an interference of the His$_x$-Myc tag with the interpretation of electron microscopy images, the His$_x$-Myc sequence was cleaved from the fusion protein with enterokinase (Roche Molecular Biochemicals) and separated on a second nickel column. Enterokinase was removed from the sample by using enterokinase-away resin (Invitrogen), following the manufacturers protocol. Recombinant BSP (10 μg/ml) was dialyzed overnight against 0.2 M ammonium formate, diluted 1:1 with 80% glycerol, sprayed onto freshly cleaved mica, dried in vacuum, and rotary shadowed with platinum/carbon as described previously (31). The replicas were floated off onto distilled water and picked up on 400-mesh copper grids. For negative staining with 0.75% uranyl formate the protein was adhered onto carbon-coated copper grids rendered hydrophilic by glow discharge (32). All samples were examined in a Zeiss EM 902 electron microscope.



tic acid to elute the oligosaccharides which were then dried in a Speed-Vac evaporator.

Release of O-Linked Oligosaccharides by Hydrazinolysis—O-Glycans were released from 20 to 200 μg of BSP by anhydrous hydrazinolysis using the O-glycan release kit (Glyco) according to Patel et al. (36). The salt-solubilized proteins in aqueous media were incubated with 2 molar hydrazine, which was subsequently removed by evaporation under reduced pressure. For the re-N-acetylation the samples were treated for 15 min at 4 °C with 2 mM acetic anhydride in ice-cold saturated barbiturate.

After desalting of the reaction mixture with the supplied ion exchange resin the glycans were dried in a Speed-Vac evaporator.

2-Aminobenzamide Labeling of Glycans—The glycans were labeled with the fluorescent dye 2-aminobenzamide according to the method described by Bigge et al. (37). The dried glycans were resuspended in 2 μl of 1 M 2-aminobenzamide in 100% acetic acid (2-aminobenzamide (Fluka) was recrystallized twice from ethanol and stored under argon at −20 °C) and 3 μl of 2 mM sodium cyanoborohydride (Sigma) in dimethyl sulfoxide. After a 2-h labeling reaction at 60 °C the samples were dotted onto chromatography paper (Schleicher & Schuell) and excessive labeling reagents were separated by chromatography in n-butanol:ethanol:water (4:1:1). The labeled glycans do not migrate under these conditions. After chromatography the application points were cut out, the labeled glycans eluted with 200–500 μl water by using centrifugal microfiltration tubes and stored at −20 °C.

Some Glycosidase Digestions—To determine the monosaccharide sequence of the N-linked oligosaccharides, the 2-aminobenzamide-labeled N-glycans were digested with specific exoglycosidases. 20 μl of each sample was incubated sequentially with neuraminidase (New England Biolabs), β-N-acetylhexosaminidase (Glyco), β-galactosidase (Glyco), and α-fucosidase (Glyco) for a total of 18–20 h at 37 °C in 50 mM sodium citrate, pH 4.5. The digests were dried in a Speed-Vac evaporator, dissolved in 75% acetonitrile in water, and used for HPLC analyses.

HPLC Analyses of 2-Aminobenzamide-labeled Oligosaccharides—A Beckman System Gold HPLC station was used together with a Shimadzu RF-10A XL fluorescence detector and the Beckman Gold Nouveau software. The excitation wavelength was 330 nm and the emission wavelength 420 nm. Normal phase HPLC was performed with a polymer-based aminophase column (aspec NH2 polymer, 5 μm, 4.6 × 250 mm). The 2-aminobenzamide-labeled oligosaccharides (20 μl) were loaded onto the column dissolved in 75% acetonitrile in water. The linear elution gradient for O-glycans ranged from 80% acetonitrile and 20% 0.25 mM ammonium formate, pH 4.5, to 20% acetonitrile and 80% 0.25 mM ammonium formate, pH 4.4, over a period of 120 min at a flow rate of 0.5 ml/min. The linear gradient used for the partially deglycosylated N-glycans started at 68% acetonitrile and 32% 50 mM ammonium formate, pH 4.4, went over 60 min to 50% acetonitrile and 50% 50 mM ammonium formate, pH 4.4, and in 3 min to 100% ammonium formate at a flow rate of 0.5 ml/min.

For anion exchange HPLC a polymer-based anion exchange column (Beckman, Q Hypersil 10, 14 μm, 4.6 × 100 mm) was used at a flow rate of 0.2 ml/min. The 2-aminobenzamide-labeled oligosaccharides were loaded onto the column. Elution buffers were H2O and 0.5 mM ammonium formate, pH 9.0 (buffer B). Sialo-N-glycans were eluted for 1 min with 0% B, for 12 min with 0–5% B, for 13 min with 5–21% B, for 25 min with 21–80% B, and for 4 min with 80–100% B. Ammonium formate buffers were prepared by adjusting 50 mM or 0.25 mM formic acid to pH 4.5 with 25% aqueous ammonium solution. Standard glycans (N-acetylhexosamines derived from bovine fetuin) and glucoside ladders that were separated under the same conditions were used to identify the different structures.

MALDI-TOF Mass Spectrometry—For peptides the matrix α-cyano-4-hydroxycinnamic acid (saturated solution in acetonitrile, 0.1% trifluoroacetic acid, 2:1) was mixed on the target 1:1 by volume with 0.5 μl of matrix solution (dihydroxy benzoic acid, 10 mg/ml H2O, acetonitrile, 2:1, v/v). The samples were dissolved in 75% acetonitrile in water. The matrix:protein mixture was spotted onto the target and allowed to air dry.

For protein analyses 5 μg of BSP was dissolved in 50 mM NH4HCO3, pH 8.0, and digested with 1 unit of PNGase F (Roche Molecular Biochemicals) for 18–20 h at 37 °C. After drying the digest in a Speed-Vac evaporator the mass of this sample and of an equal aliquot of undigested protein was determined on a Reflex III mass spectrometer (Bruker).

N-Glycans were analyzed by reflection MALDI-TOF mass spectrometry using dihydroxy benzoic acid (10 mg/ml H2O, acetonitrile, 2:1, v/v). The samples were dissolved in 50% acetonitrile in water, and 1 μl was spotted onto the target. A pulsed laser beam (nitrogen laser, 28.5 kV in reflectron mode) and 22 kV in PSD mode) fractured the sample. Spectra were obtained in the positive ion mode. Acceleration and reflector voltages were set to 20 kV (28.5 kV in PSD mode) and 22 kV in PSD mode, respectively. The molecular parent ion was isolated with a pulsed field by deflection (28.5 kV in PSD mode) and 22 kV (30 kV in PSD mode), respectively. Positive ion mode. Acceleration and reflector voltages were set to 20 kV (28.5 kV in PSD mode) and 22 kV in PSD mode, respectively. The molecular parent ion was isolated with a pulsed field by deflection (28.5 kV in PSD mode) and 22 kV (30 kV in PSD mode), respectively.

Partially Methylated Alditol Acetates—O-Linked glycans on recombinant BSP were liberated from the protein by incubation in a mixture of powder NaOH in dimethyl sulfoxide and methanol/methylene chloride. After overnight reaction at ambient temperature the methylated glycans were extracted with chloroform:water and the organic phase was washed three times with water prior to drying in a stream of nitrogen. Methylated glycans were either directly analyzed by MALDI-TOF mass spectrometry or converted into the partially methylated alditol acetates to obtain information on the glycosidic structures. For this purpose the oligosaccharides were cleaved by mild hydrolysis, followed by reduction and re-N-acetylation according to Levery et al. (39). The monosaccharide derivatives were analyzed by gas chromatography electron impact ionization mass spectrometry (MD800, Fisons, Manchester, United Kingdom) on a 15-m DB5ms capillary column heated from 50 to 250 °C (7.5 °C/min). The partially methylated alditol acetates were registered by monitoring the total ion current and single ions at m/z 118 (hexitole) and 159 (N-acetylated hexosamines). The substation isomers were finally identified via their primary fragmenta- tion patterns.

Localization of O-Glycosylation Sites—About 100 μg of recombinant BSP was treated with 0.1 M aqueous trifluoroacetic acid at 80 °C (1 h) to cleave sialic acids. The asialo derivative was extensively digested with a mixture of β-galactosidase (Glyco) and β-hexosaminidase (Glyco) in order to reduce O-glycans to the level of the core-GalNAc. After desalting on NAP-5 column (Amersham Pharmacia Biotech), aliquots of the partially deglycosylated protein were proteolytically cleaved by sequential treatment with trypsin (sequencing grade, Promega, 1–2% enzyme:substrate in 50 mM NH4HCO3, pH 8.0) for 2–4 h at 37 °C and V8 protease (sequencing grade, Roche Molecular Biochemicals, 1–2% enzyme:substrate in 50 mM NH4HCO3, pH 8.0) for 16 h at 37 °C. After digestion the protein was separated by reverse-phase HPLC (AC18, 2 × 150 mm, Beckman-Coulter) and fractions were dried by Speed-Vac evaporation. A peptide mapping was performed by reflection MALDI-TOF mass spectrometry (and glyco) peptides were identified via their monoisotopic masses. Sequence analysis of glyco/peptides was performed by MALDI-TOF mass spectrometry in the PSD mode (see above).

RESULTS

Isolation of Human Bone BSP by Recombinant Expression and by Purification from Bone Extracts—BSP was recombinantly expressed in the human embryonic kidney cell line EBNA-293 as a secreted His6-Myc fusion protein and purified to homogeneity by affinity chromatography using a nickel column. The yield of recombinant BSP per liter of serum-free supernatant was about 3 mg. After SDS-PAGE and silver staining the protein was detected as a broad band between 70 and 80 kDa (Fig. 1), the His6-Myc tag contributing 5 kDa. BSP was also isolated from human bone powder. The noncollagenous proteins were extracted with EDTA at pH 7.5 and BSP purified using standard chromatography methods giving a distinct band at 70 kDa in SDS-PAGE (Fig. 1). The different sizes and color yields after SDS-PAGE and silver staining of recombinant and bone-derived BSP presumably reflect differences in their post-transla- tion modifications (see below).

Recombinant BSP Consists of a Globule Linked to a Thread-like Structure—Recombinant BSP was visualized by electron microscopy after rotary shadowing (Fig. 2A) or negative staining (Fig. 2B). For this purpose the His6-Myc tag had been

Characterization of Human Bone Sialoprotein (BSP) 36841

http://www.jbc.org/content/267/24/36841.full.pdf+html

http://www.jbc.org/content/267/24/36841.full.pdf+html
removed by cleavage with enterokinase and separation on a second nickel column. The protein is a monomer possessing a globular structure with a diameter of 10 ± 1 nm that is linked to a thread-like structure of 25 ± 6 nm length. The globule is likely to correspond to the C-terminal part of recombinant BSP that appears to be devoid of glycans (see below) and the thread-like structure to the highly glycosylated N-terminal part of the protein.

**Recombinant BSP and Bone-derived BSP Have a Similar Secondary Structure**—Circular dichroism spectra of BSP were characteristic for proteins with a low proportion of α-helix and β-sheet elements and a high content of random coil structure (Fig. 3). This was underlined by analyses of the CD spectra that revealed 5% α-helix, 32% β-sheet, 17% β-turn, and 46% random coil structure for bone-derived BSP. The CD spectra and thereby the secondary structures of recombinant BSP lacking the His6-Myc tag and bone-derived BSP were similar. This shows that BSP can be isolated from bone without a loss of structure. Recombinant BSP that was treated with 6 M Gdn-HCl showed a marked unfolding. CD-spectra for native and renatured recombinant His6-Myc-BSP were nearly identical, showing that the protein can be refolded completely by dialysis against a physiological buffer. Comparison between the spectra of His6-Myc-tagged and non-tagged BSP showed that the His6-Myc sequence contributes some secondary structure (Fig. 3). Inclusion of 2 mM CaCl2 in the buffer had only a minor influence on the spectra and the effect was not reversible after the addition of EDTA, while proteolytic cleavage of recombinant BSP with the calcium-independent protease α-chymotrypsin was slightly enhanced in 2 mM CaCl2 (results not shown).

**Cell Binding to BSP Is Reversibly Diminished by Denaturation**—To investigate the influence of BSP conformation on cell binding, recombinant BSP was coated onto 96-multiwell plates either in the native structure (dissolved in 50 mM Tris-HCl, pH 8.0), in denatured form (after dialysis against 6 M Gdn-HCl), or in renatured form (after dialysis against 6 M Gdn-HCl followed by dialysis against 50 mM Tris-HCl, pH 8.0). Cell adhesion assays were performed using the human adenocarcinoma cell line MCF-7. The cells showed the highest binding to native recombinant BSP, while adhesion was decreased when using denatured BSP and largely restored when BSP was renatured (Fig. 4), in parallel to the reversible loss and restoration of secondary structure.

**Glycosylation of BSP—MALDI-TOF mass spectrometry**—Analyses of recombinant BSP revealed a broad peak between 40 and 75 kDa with an average molecular mass of 57 kDa, indicating a heterogenous glycosylation. The His6-Myc tag contributes 5 kDa to these values. For bone-derived BSP the peak ranged from 40 to 60 kDa with an average molecular mass of 49 kDa (Fig. 5). By comparison with the theoretical protein molecular mass for human BSP (33.6 kDa), deduced from the cDNA sequence (2), a mass contribution of 30 or 40% by post-translational modifications could be calculated for bone-derived BSP and recombinant BSP, respectively. The MALDI-TOF results further showed that BSP has an aberrant migration behavior in SDS-PAGE. After digestion of the protein with PNGase F, an enzyme that cleaves nearly all types of N-glycans from asparagine residues, the molecular mass shifted from 57 to 52 kDa for recombinant BSP and from 49 to 43 kDa for bone-derived BSP (Fig. 5). Accordingly, the N-glycans on recombinant BSP have a total mass of 5 kDa and make up 20% of the post-translational modifications. In bone the N-glycans have a total mass of 6 kDa and make up 40% of the post-translational modifications on BSP. After PNGase F treatment the BSP peaks were still polydisperse with masses...
The Composition of Complex-type N-Glycans on Recombinant BSP Is Different from That on Bone-derived BSP—N-Glycans were enzymatically cleaved from asparagine residues of BSP with peptide N-glycosidase F and labeled with the fluorescent dye 2-amino benzamide. Sialo-structures were separated on a normal phase HPLC column and identified by sequential digestion with different exoglycosidases (neuraminidase, endoglycosidase H, an enzyme that cleaves only high mannose type N-glycans, gave no detectable mass shifts (results not shown), showing that BSP contains only N-glycans of the complex-type.

The Composition of Complex-type N-Glycans on Recombinant BSP—N-Glycans were tetraantennary structures predominantly (57%) of the total sialo-N-glycans. After digestion with neuraminidase the asialo-N-glycans were enzymatically released from BSP by treatment with PNGase F and labeled with the fluorescent dye 2-amino benzamide. Sialo-structures were separated on a normal phase HPLC column and identified by sequential digestion with different exoglycosidases (neuraminidase, endoglycosidase H, an enzyme that cleaves only high mannose type N-glycans, gave no detectable mass shifts (results not shown), showing that BSP contains only N-glycans of the complex-type.

The Composition of Complex-type N-Glycans on Recombinant BSP Is Different from That on Bone-derived BSP—N-Glycans were enzymatically cleaved from asparagine residues of BSP with peptide N-glycosidase F and labeled with the fluorescent dye 2-amino benzamide. Sialo-structures were separated on a normal phase HPLC column and identified by sequential digestion with different exoglycosidases (neuraminidase, endoglycosidase H, an enzyme that cleaves only high mannose type N-glycans, gave no detectable mass shifts (results not shown), showing that BSP contains only N-glycans of the complex-type.

The Composition of Complex-type N-Glycans on Recombinant BSP Is Different from That on Bone-derived BSP—N-Glycans were enzymatically cleaved from asparagine residues of BSP with peptide N-glycosidase F and labeled with the fluorescent dye 2-amino benzamide. Sialo-structures were separated on a normal phase HPLC column and identified by sequential digestion with different exoglycosidases (neuraminidase, endoglycosidase H, an enzyme that cleaves only high mannose type N-glycans, gave no detectable mass shifts (results not shown), showing that BSP contains only N-glycans of the complex-type.

The Composition of Complex-type N-Glycans on Recombinant BSP Is Different from That on Bone-derived BSP—N-Glycans were enzymatically cleaved from asparagine residues of BSP with peptide N-glycosidase F and labeled with the fluorescent dye 2-amino benzamide. Sialo-structures were separated on a normal phase HPLC column and identified by sequential digestion with different exoglycosidases (neuraminidase, endoglycosidase H, an enzyme that cleaves only high mannose type N-glycans, gave no detectable mass shifts (results not shown), showing that BSP contains only N-glycans of the complex-type.

The Composition of Complex-type N-Glycans on Recombinant BSP Is Different from That on Bone-derived BSP—N-Glycans were enzymatically cleaved from asparagine residues of BSP with peptide N-glycosidase F and labeled with the fluorescent dye 2-amino benzamide. Sialo-structures were separated on a normal phase HPLC column and identified by sequential digestion with different exoglycosidases (neuraminidase, endoglycosidase H, an enzyme that cleaves only high mannose type N-glycans, gave no detectable mass shifts (results not shown), showing that BSP contains only N-glycans of the complex-type.
O-Glycans were released from BSP by anhydrous hydrazinolysis, labeled with the fluorescence dye 2-aminobenzamide and separated on a normal phase HPLC column. Eight different mucin-type structures could be determined by comparison of the HPLC peaks with standard O-glycans (derived from glycophorin A or mucin 1), by sequential exoglycosidase digestion of single peaks, and through determining the masses by MALDI-TOF mass spectrometry of the methylated derivatives. The percentage of the structures was calculated from the peak areas. Comparative analyses of the O-glycans (Fig. 7, Table II) revealed both similarities and differences between recombinant BSP and BSP isolated from bone. Core 1 (Galβ(1→3)GalNAc) structures were visible in both preparations, whereas core 2 (GlcNAcβ(1→3)(Galβ(1→3)3GalNAc) structures were absent in bone-derived BSP. The relative proportion of the structures differed with the main component of bone-derived BSP O-glycans being peak 8, a short disialylated core 1 structure, whereas in recombinant BSP mono- or disialylated core 1 or core 2 structures each contributed about 20% of the total O-glycans. Only a small proportion (12% in bone-derived BSP, 5% in recombinant BSP) lacked terminal N-acetylneuraminic acids. Multiplication of the number of the terminal NeuAc with the relative proportion of each glycan species (Table II) showed that the O-glycans on bone-derived BSP carry more NeuAc (160) than those on recombinant BSP (125). Fucosylated structures were not detected in the HPLC and methylation analyses confirmed the absence of 2-fucosylated O-glycans. In addition to 1,3,5-tri-O-acetyl-galactitol and 1,4,5-tri-O-acetyl-N-acetylgulosaminitol, the major partially methylated alditol acetates, methylation analysis indicated the presence of 3,6-branched galactose and 4,6-branched N-acetylgulosamin as minor products. Core 2 structures dominated over core 1 structures on recombinant BSP according to methylation analyses.

Recombinant BSP Contains Four N-Glycosylation Sites and Eight O-Glycosylation Sites Were Identified—Sequence analyses of the human BSP cDNA revealed four potential N-glycosylation sites (Table II) and eight O-glycosylation sites (Fig. 8). As a consensus sequence for O-glycosylation has not been established we determined part of the O-glycosylation sites on recombinant BSP experimentally. The O-glycans were enzymatically cleaved down to the core GalNAc followed by partial proteolytic cleavage of the protein. The

| peak | structure | % rec. BSP | % bone BSP | average mass of molecular ions [M+Na] |
|------|-----------|------------|------------|--------------------------------------|
| II   | Galβ(1→4)GlcNAcβ(1→2)Man(β1→6)Manβ(1→3)     | 17%        | 11%        | 1810.3                               |
|      | Galβ(1→4)GlcNAcβ(1→2)Manβ(1→3)              |            |            | 2013.5                               |
|      | (GlcNAc)                                      |            |            |                                      |
| III  | Galβ(1→4)GlcNAcβ(1→2)Man(β1→6)               | 12%        | 57%        | 2175.9                               |
|      | Galβ(1→4)GlcNAcβ(1→3)Man(β1→3)              |            |            |                                      |
| IV   | Galβ(1→4)GlcNAcβ(1→2)Man(β1→6)              | 44%        | 8%         | 2541.0                               |
|      | Galβ(1→4)GlcNAcβ(1→3)Man(β1→3)              |            |            |                                      |

---

**TABLE I**

Major BSP asialo N-glycans

---

*Peak number obtained after separation of the N-glycans on normal phase HPLC.*

*Structures derived from HPLC analyses of standard N-glycans and mass spectrometry.*

*Percentage of the different asialo structures relative to the total N-glycans, calculated from the peak areas.*

*Average masses of the molecular ions (Na-adducts) obtained by MALDI-TOF mass spectrometry.*
masses of the peptides were determined by MALDI-TOF mass spectrometry and selected peptide ions were sequenced by PSD MALDI-TOF mass spectrometry. The maximal number of O-glycosylation sites per peptide was deduced by comparing the experimentally determined and calculated masses (results not shown) and related to the threonines with the highest probability of being O-glycosylated in terms of their sequence context (Fig. 8). Although the peptide fragments did not span the whole BSP sequence (the intervening sequences are rich in glutamate and extensively cleaved by V8 protease), we could determine eight O-glycosylation sites on recombinant BSP, five sites on peptide 261–279, and three sites on peptide 151–180 (the third could not be localized to a certain threonine within the peptide sequence). Identical peptides with different numbers of GalNAc residues, e.g. peptide 261–279, with one to five GalNAc residues were found demonstrating a microheterogeneity of BSP O-glycosylation. Cleavage products of the C-terminal region of BSP (288–294, 336–351) were not detected as glycosylated peptides.

Bone-derived BSP Has a Higher Affinity for Hydroxyapatite Than Recombinant BSP—Analyses of the glycosylation pattern revealed differences between recombinant and bone-derived BSP. Therefore we investigated whether these differences influence the functional properties of the molecule. Many results point towards a role for BSP as a nucleator for hydroxyapatite crystals and therefore the affinity for hydroxyapatite was examined. Equal amounts of recombinant BSP and bone-derived BSP were applied to a hydroxyapatite column and eluted with a linear gradient from 10 mM to 0.5 M sodium phosphate, pH 7.2. Fractions were collected and analyzed for their BSP content by Western blotting (Fig. 9). The sodium phosphate concentration needed for BSP elution served as a parameter for hydroxyapatite affinity. BSP isolated from bone had the strongest affinity for hydroxyapatite and was eluted with 295–345 mM sodium phosphate (fractions 12–14). Recombinant BSP was eluted significantly earlier with 220–270 mM sodium phosphate (fractions 9–11).

**DISCUSSION**

Studies on the structure and function of BSP are hampered by the difficult isolation of the protein from bone, where it is entrapped in the hydroxyapatite. Usually strongly denaturing conditions such as 6 M Gdn-HCl or formic acid were used to isolate BSP (3–5, 40). Prokaryotic expression systems were not

### Table II
BSP O-glycans

| peak | structure | percentage | pseudomolecular mass M+Na measured for permethylated glycans (average masses) |
|------|----------|------------|---------------------------------------------------------------------------|
| 1    | GalNAc   | 2%         | n. d.                                                                     |
| 2    | Gal(91-3)GalNAc | 2% | 518.1                                                                   |
| 3    | Gal(91-4)GlcNAc(91-6)Gal(91-3)GalNAc | 8% | 967.5                                                                   |
| 4    | NeuAc(α2-3)Gal(91-3)GalNAc | 24% | 879.4                                                                   |
| 5    | NeuAc(α2-6)Gal(91-3)GalNAc | 5% | n. d.                                                                   |
| 6    | NeuAc(α2-3)Gal(91-4)GlcNAc(91-6)Gal(91-3)GalNAc | 20% | 1328.7                                                                  |
| 7    | NeuAc(α2-3)Gal(91-4)GlcNAc(91-6)NeuAc(α2-3)Gal(91-3)GalNAc | 18% | 1689.8                                                                  |
| 8    | NeuAc(α2-6)NeuAc(α2-3)Gal(91-3)GalNAc | 20% | 1240.7                                                                  |

**a** Peak number obtained after separation of the O-glycans on normal phase HPLC.

**b** Structures derived from HPLC analyses of standard O-glycans and methylation analyses.

**c** Relative proportion of the different structures, calculated from the peak areas.

**d** Mass of permethylated O-glycans obtained after methylation, β-elimination and MALDI-TOF mass spectrometry.

ND, not determined.
able to express full-length recombinant BSP (20). Here we show that full-length recombinant human BSP can be produced in high yields by a human cell line.

Recombinant human BSP was detected by MALDI-TOF mass spectrometry as a broad peak between 40 and 75 kDa with an average molecular mass of 57 kDa being nearly twice the molecular mass deduced from the cDNA sequence (33.6 kDa, (2)). Human bone-derived BSP that was isolated under non-denaturating conditions had a smaller mass range (40–60 kDa) with an average mass of 49 kDa reflecting the lower content of post-translational modifications (30%) compared with the recombinant protein (40%).

In SDS-PAGE recombinant as well as bone-derived BSP showed an aberrant migration. Recombinant His$_6$-Myc-BSP mass spectrometry was as a broad band between 70 and 80 kDa, whereas BSP isolated from human bone under native conditions appeared as a distinct band at 70 kDa. BSP isolated from human bone under denaturing conditions was earlier described as an 80-kDa band while the sizes of BSPs from different other species were between 55 and 82 kDa (4, 5, 7, 19, 40, 41). This might partially be due to different migration depending on the polycrylamide gel concentrations used. However, for bovine BSP a molecular mass of 57.3 kDa was determined by sedimentation-equilibrium centrifugation (3) which is clearly different from the 49 kDa determined here for BSP from human bone. As primary sequences are very similar, post-translational modifications are likely to cause the different molecular masses of BSP from different species.

Recombinant BSP was shown to have a globular structure linked to a thread-like structure of a total length of 35 ± 7 nm by rotary shadowing or negative staining. As the C terminus of the protein was shown to be free of glycans we suggest that this part of the protein forms a globular structure, whereas the highly glycosylated regions are thread-like due to a lack of secondary structure. BSP isolated from bovine bone under denaturing conditions was described as an extended rod having a core with an average length of 40 nm after rotary shadowing (3), similar to the recombinant protein in terms of size and structure. The observed globular structure in a part of BSP is in apparent disagreement with recent results from Fisher et al. (42) who by one-dimensional proton NMR characterized recombinant BSP as being completely unstructured and flexible in solution. At present the reason for this discrepancy is not clear.

FIG. 8. Glycosylation sites of recombinant BSP. O-Glycosylation sites were determined by digesting the O-glycans down to the core GalNAc, followed by proteolytic fragmentation (V8 and trypsin), MALDI-TOF mass spectrometry, and PSD sequencing of selected peptide fragments (framed and numbered with the corresponding amino acids). The His$_6$-Myc fusion peptide sequence is printed in italics. The maximal number of O-glycosylation sites was deduced from the masses of the peptide fragments and the threonines with the highest probability of being O-glycosylated in terms of their sequence context are printed in bold letters and marked with a circle. The O-glycosylation sites predicted with the NetOGlyc program are in shaded boxes. N-Glycosylated asparagines are printed in bold and marked with a triangle.

Investigation of the secondary structure elements of recombinant BSP by circular dichroism spectroscopy revealed a small but significant proportion of α-helix (5%) and β-sheet elements (32%) with most of the structure in a random coil. BSP could be completely denatured by the addition of 6 M Gdn-HCl. In vivo, BSP is entrapped in the hydroxyapatite matrix of bone. Interestingly, when we isolate BSP from human bone under native conditions, thus avoiding the usually used strongly denaturing conditions such as 6 M Gdn-HCl or formic acid, the secondary structure was very similar to the recombinant non-tagged protein. The His$_6$-Myc tag contributed some secondary structure to the recombinant protein as shown by a different CD spectrum compared with non-tagged BSP. The addition of calcium and phosphate ions to the recombinant BSP solution did not change the secondary structure as analyzed by CD spectroscopy.

The in vitro binding of recombinant BSP to the human adenocarcinoma cell line MCF-7 showed a strong dependence on the intact conformation of the protein with significantly decreased binding to denatured protein. Thus, cell adhesion results that have been obtained with recombinant BSP produced in Escherichia coli or with irreversibly denatured protein may not fully reflect the in vivo situation.

N-Glycan and O-glycan structures on human bone-derived BSP and on BSP recombinantly expressed in the human embryonic kidney cell line EBNA-293 were identified. Cleavage of all N-glycans with PNGase F resulted in a mass shift of 5 kDa for recombinant BSP and of 6 kDa for bone-derived BSP whereas digestion with endoglycosidase H had no effect on the size of the protein, indicating that BSP contains only complex-type N-glycans. Analyses of the N-glycans by HPLC and MALDI-TOF mass spectrometry lead to the identification of different complex-type structures with 2–4 antennae that could carry 1–4 fucose residues. All BSP N-glycans have a core fucose and biantennary structures were also present as bisecting glycans (Table I). The high diversity of the N-glycans resulted from the numerous combination possibilities of the identified siafo- and asialo-structures. All N-glycans were found both on recombinant and on bone-derived BSP. Differences

FIG. 9. Hydroxyapatite affinity of BSP. 50 μg of recombinant (A) and bone-derived (B) BSP were loaded onto a hydroxyapatite column and eluted with a linear gradient from 10 mM to 0.5 M sodium phosphate. 20 μl of each fraction was analyzed for its BSP content by Western blotting using the monoclonal antibody. Lane A contains the flow-through; lane B, the wash fraction. Fractions with the highest BSP concentrations are boxed. They correspond to sodium phosphate concentrations of 220–270 mM (9–11) for recombinant BSP and to 295–345 mM (12–14) for bone BSP.
were observed only in the relative proportions of the structures. The main part of the bone-derived BSP N-glycans were triantennary (57%) whereas in recombinant BSP tetraantennary structures were predominant (44%) (Table I). On bone-derived BSP over half of the glycans carried three NeuAc, most likely representing the major triantennary structures with one terminal NeuAc per arm. On recombinant BSP mono-, di-, tri-, and tetrasialo structures were equally distributed with 20–30% each. As most of the recombinant BSP N-glycans were tetraantennary, peak areas containing more than one maximum might represent tetraantennary structures with one, two, or three terminal NeuAc. When multiplying the percentages of the structures calculated from the peak areas with the number of the corresponding NeuAc, resulting in the hypothetical N-glycan charge (43) this number was only slightly different for recombinant BSP (Z = 223) and bone-derived BSP (Z = 228). We are not aware of any systematic study investigating the repertoire of glycosyltransferases present in osteoblasts. The extracellular matrix protein BM-40 (osteonectin), that, like BSP, comprises about 15% of the total non-collagenous proteins in bone, was shown to have one used N-glycosylation site and carries only high-mannose-type N-glycans when isolated from bone. However, BM-40 that was isolated from platelets or from serum-free supernatants of different osteosarcoma cell lines, an endothelial cell line and the human embryonic kidney cell line EBNA-293 was reported to have only N-glycans of the complex-type (44). Thus, the local surroundings of the respective asparagines in BSP and BM-40 are compatible with the formation of complex-type N-glycans from the high-mannose precursors. The fact that BM-40 is not modified to complex-type N-glycans by osteoblasts, while BSP is, therefore points to a different osteoblast population being responsible for the synthesis of each protein or to a differential sorting during their transit through the endoplasmic reticulum and Golgi apparatus.

Analyses of the O-glycans by HPLC and MALDI-TOF mass spectrometry led to the identification of eight different mucin-like O-glycan structures. Core 1-derived O-glycans were detected on both recombinant and bone-derived BSP whereas core 2-derived structures were found only on the recombinant protein (Table II). This is presumably due to an inability of osteoblasts to synthesize core 2 resulting from a possible lack of β-N-acetylglucosaminyltransferase activity. The activity of this enzyme in osteoblasts has not been described. Differences occurred also in the relative proportions of the structures with the predominant part (66%) of bone-derived BSP O-glycans being a disialylated core1 structure whereas mono- or disialylated core 1 or core 2 structures are equally represented on recombinant BSP. Fucosylated O-glycans were not found and the proportion of non-sialylated structures (12% for recombinant BSP, 5% for bone-derived BSP) was low, the O-glycans on bone-derived BSP having about 25% more NeuAc than those on recombinant BSP (Table II).

In a review by Midura et al. (45) some preliminary results on O-glycan structures of BSP isolated from the rat osteosarcoma cell line UMR-106 were described. The five different core 1- or core 2-derived O-glycan structures on rat BSP were also identified for human BSP in this work. A core 6-derived structure found by Midura et al. (45) was absent on human BSP. Also sulfated N- and O-glycans (6, 45) were not found on human BSP.

Interestingly, short highly sialylated core 1 structures, similar to the main O-glycans on bone-derived BSP are frequently found on mucins in tumor cells (46) because of a lack of the core 2 enzyme or an increased sialyltransferase activity. Whether these O-glycans, common to tumor cells and bone-derived BSP, are somehow connected with a possible function for BSP in breast cancer and the development of bone metastases remains to be determined. The present investigation provides a basis to study whether BSP expressed in breast tumors differs in structure from bone-derived BSP.

We could identify eight O-glycosylation sites by MALDI-TOF mass spectrometry analyses of partially deglycosylated and proteolytically fragmented recombinant BSP peptides (Fig. 8). From the identification of the same peptides with different numbers of bound O-glycans we concluded that these O-glycosylation sites can be fully glycosylated, but are not modified at all times. Thus, as many other glycoproteins, BSP shows a site heterogeneity. The O-glycosylation sites were related to threonines that have the highest possibility of being O-glycosylated deduced from their sequence context. Using this method seven of the eight glycosylation sites could be localized, only the third glycosylation site on peptide 151–180 could not be determined.

Out of a total of 53 serines and threonines the NetOgly program predicted 11 potential O-glycosylation sites. The underlying algorithm is based on statistical analyses of 299 known mucin-like O-glycosylation sites and 2516 non-glycosylated serines and threonines out of a total of 60 characterized glycoproteins, including the sequence context and surface accessibility of the amino acids (47). When comparing the experimentally obtained data with the NetOgly predictions, four O-glycosylation sites were identical, three threonines, not predicted by NetOgly were shown to carry O-glycans and six serines or threonines described as being glycosylated by NetOgly were not included in the proteolytically obtained peptides (Fig. 8). Although the investigated peptides did not span the whole BSP sequence, important O-glycosylated threonine clusters were detected. Considering the two non-glycosylated peptides of the C-terminal region (288–294, 336–351) and that NetOgly predicts no O-glycosylation sites from amino acids 283–351, it is very likely that the C terminus of BSP is free of glycans. We also can exclude the presence of chondroitin sulfate and dermatan sulfate chains on recombinant BSP as digestion with chondroitinase ABC did not affect the electrophoretic mobility in SDS-PAGE (results not shown). Only BSP isolated from rabbit calvaria has been identified as a keratan sulfate proteoglycan (48), whereas BSP from other species did not contain any glycosaminoglycan chains (5, 49).

The affinity for hydroxyapatite was investigated to find out whether the differences in glycosylation between recombinant BSP and bone-derived BSP influence the functional properties of the molecule. Binding to a hydroxyapatite column, as investigated by elution with a linear sodium phosphate gradient, was significantly stronger for bone-derived BSP than for the recombinant protein. The different affinities for hydroxyapatite are most probably caused by the higher charge contributed by the higher NeuAc content of bone-derived BSP.

In conclusion we have produced full-length recombinant human BSP in a eukaryotic expression system and purified the protein from human bone under conditions retaining a native structure. Our results show that both proteins are post-translationally N- and O-glycosylated although in a different manner. The recombinant BSP can serve as an improved tool for the functional investigation of BSP. The characterization of the glycan structure sets the basis for analyzing the difference between bone-derived and tumor-derived BSP.

Acknowledgments—We thank Dr. F.-P. Armbruster and Dr. M. Karmatschek, Immundiagnostik AG, Bensheim, Germany, for kindly

---

2 B. Kaufmann, personal communication.
providing the bone powder and the BSP monoclonal antibody as well as for helpful discussions.

REFERENCES

1. Herrig, G. M. (1972) in The Biochemistry and Physiology of Bone (Bourne, G. H., ed) Vol. 1, pp. 127–189, Academic Press, New York
2. Fisher, L. W., McIride, O. W., Termine, J. D., and Young, M. F. (1990) J. Biol. Chem. 265, 2347–2351
3. Franzen, A., and Heinegärd, D. (1985) Biochem. J. 232, 715–724
4. Fisher, L. W., Hawkins, G. R., Tuross, N., and Termine, J. D. (1987) J. Biol. Chem. 262, 9702–9708
5. Fisher, L. W., Whiston, S. W., Avioli, L. V., and Termine, J. D. (1988) J. Biol. Chem. 263, 12723–12727
6. Midura, R. J., McQuillan, D. J., Benham, K. J., Fisher, L. W., and Hascall, V. C. (1990) J. Biol. Chem. 265, 5285–5291
7. Ek-Rylander, B., Flores, M., Wendel, M., Heinegärd, D., and Andersson, G. (1994) J. Biol. Chem. 269, 14853–14856
8. Oldberg, A., Franzen, A., Heinegärd, D., Pierschbacher, M., and Ruoslabäki, E. (1988) J. Biol. Chem. 263, 19433–19436
9. Oldberg, A., Franzen, A., and Heinegärd, D. (1988) J. Biol. Chem. 263, 19430–19432
10. Hultenby, K., Reinholdt, F. P., Norgard, M., Oldberg, A., Wendel, M., and Heinegärd, D. (1994) Eur. J. Cell Biol. 63, 230–239
11. Ingram, R. T., Clarke, B. L., Fisher, L. W., and Fitzpatrick, L. A. (1993) J. Bone Miner. Res. 8, 1019–1029
12. Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D., and Robey, P. G. (1991) Calcif. Tissue Int. 49, 421–426
13. Chenu, C., and Delmas, P. D. (1992) J. Bone Miner. Res. 7, 47–54
14. Waltregny, D., Bellahcene, A., de Leval, X., Florkin, B., Weidle, U., and Castronovo, V. (2000) J. Bone Miner. Res. 15, 834–843
15. Grzesik, W. J., and Robey, P. G. (1994) J. Bone Miner. Res. 9, 487–496
16. Flores, M. E., Norgard, M., Heinegärd, D., Reinholdt, F. P., and Andersson, G. (1995) Exp. Cell Res. 210, 536–539
17. Ross, F. P., Chappel, J., Alvarez, J. I., Sander, D., Butler, W. T., Farach-Carson, M. C., Mintz, K. A., Robey, P. G., Teitelbaum, S. L., and Oldfield, E. (1993) J. Cell Biol. 120, 9901–9907
18. Oldberg, A., Jirskog-Hed, B., Axelsson, S., and Heinegärd, D. (1989) J. Cell Biol. 109, 3183–3186
19. Heinegärd, D., and Oldberg, A. (1989) FASEB J. 3, 2042–2051
20. Stubbs, J. T., III, Mintz, K. A., Eanes, K. D., Torchia, D. A., and Fisher, L. W. (1997) J. Bone Miner. Res. 12, 1210–1222
21. Hunter, G. K., and Goldberg, H. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8562–8565
22. Chen, Y., Bal, B. S., and Gorski, J. P. (1992) J. Biol. Chem. 267, 24871–24878
23. Cowles, E. A., DeRome, M. E., Pastizzo, G., Bradley, L. L., and Gronowitz, G. A. (1998) Calcif. Tissue Int. 62, 74–82
24. Auh, J. E., Gupta, A., Zarngbi, R., and Rossant, J. (1995) Bone 17, 558 (Abstr. 3a)
25. Woitge, H. W., Pecherstorfer, M., Horn, E., Keck, A. V., Diel, I. J., Bayer, P., Ludwig, H., Ziegler, R., and Seibel, M. J. (2001) Br. J. Cancer 84, 344–351
26. Diel, I. J., Solomayer, E. F., Seibel, M. J., Pfeilschifter, J., Miesenbacher, H., Gollan, C., Pecherstorfer, M., Conrad, R., Kehr, G., Boehm, E., Armbruster, F. P., and Bastert, G. (1999) Clin. Cancer Res. 5, 3914–3919
27. Kohfeldt, E., Maurer, P., Vannahme, C., and Timpl, R. (1997) FEBS Lett. 414, 557–561
28. Karmatschek, M., Maier, I., Seibel, M. J., Woitge, H. W., Ziegler, R., and Armbruster, F. P. (1997) Clin. Chem. 43, 2076–2082
29. Laemmli, U. K. (1970) Nature 227, 680–685
30. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
31. Engel, J., and Furthmayr, H. (1987) Methods Enzymol. 154, 3–78
32. Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rhode, H., and Timpl, R. (1981) J. Mol. Biol. 150, 97–120
33. Gill, S. C., and Hippen, P. H. (1989) Anal. Biochem. 182, 319–326
34. Provencher, S. W., and Glöckner, J. (1981) Biochemistry 20, 33–37
35. Packer, N. H., Lawson, M. A., Jardine, D. R., and Redmond, J. W. (1998) Glycobiol. 15, 737–747
36. Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A., and Parekh, R. (1993) Biochemistry 32, 679–693
37. Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M., and Parekh, R. B. (1995) Anal. Biochem. 229, 229–238
38. Anumula, K. R., and Taylor, T. B. (1992) Anal. Biochem. 203, 101–108
39. Levery, S. B., and Hakomori, S. (1987) Methods Enzymol. 138, 13–25
40. Zhang, G., Domenici, C., Goldberg, H. A., Wranas, L. J., and Sodek, J. (1979) J. Biol. Chem. 254, 3186–3190
41. Kebbel, G. H., Vorlop, and J., Haupt, H. (1996) Glycoconj. J. 15, 681–687
42. Babski-Told, M., Dolder, W. M., and Seibel, M. J. (2001) Glycoconj. J. 15, 637–642
43. F Gore, C. N., and Seibel, M. J. (1996) Glycoconj. J. 15, 637–642
44. Uphold, C., and Seibel, M. J. (1996) Glycoconj. J. 15, 637–642
45. McQuillan, D. J., Richardson, M. D., and Bateman, J. F. (1995) Bone 17, 558 (Abstr. 3a)
