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Characterization of Osteocalcin (BGP) and Matrix Gla Protein (MGP) Fish Specific Antibodies: Validation for Immunodetection Studies in Lower Vertebrates

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Abstract. In fish species the basic mechanisms of bone development and bone remodeling are not fully understood. The classification of bone tissue in teleosts as cellular or acellular and the presence of transitional states between bone and cartilage and the finding of different types of cartilage in teleosts not previously recognized in higher vertebrates emphasizes the need for a study on the accumulation of the Gla-containing proteins MGP and BGP at the cellular level. In the present study, polyclonal antibodies developed against BGP and MGP from *A. regius* (a local marine teleost fish) and against MGP from *G. galeus* (a Pacific Ocean shark), were tested by Western blot for their specificity against BGP and MGP from several other species of teleost fish and shark. For this purpose we extracted and purified both proteins from various marine and freshwater teleosts, identified them by N-terminal amino acid sequence analysis and confirmed the presence of gamma-carboxylation in the proteins with the use of a stain specific for Gla residues. Each antibody recognized either BGP or MGP with no cross-reaction between proteins detected. All purified fish BGPs and MGPs tested were shown to be specifically recognized, thus validating the use of these antibodies for further studies.

Matrix Gla Protein (MGP) and Bone Gla Protein (BGP, osteocalcin) belong to the family of vitamin K-dependent (VKD), γ-carboxyglutamic acid (Gla)-containing proteins that have been unequivocally associated with bone formation and mineralization [1–4], and more recently, with vascular calcification [5–10].

MGP is a 10–15 kDa-secreted protein, containing 4–5 residues (depending on the species) of the Ca^{2+} binding Gla residue [11–15] while BGP is a small secreted protein with approximately 6 kDa molecular weight and includes three Gla residues. Although there is little information about the regulation of expression of these proteins in teleosts, BGP in teleost fish has been shown to be associated with bone-like mineralized tissues present in branchial arches, jaw, vertebra and scales [15, 16] while MGP was only recently found to accumulate, mainly in the extracellular matrix of calcified cartilage [15]. In this previous work, the MGP gene was found to be predominantly expressed in chondrocytes from branchial arches, with no expression detected in the different bone-like mineralized tissues analyzed while BGP mRNA was mainly located in bony tissues associated with osteoblast-like cells, as expected [15]. As previously seen in mammals and *Xenopus*, MGP mRNA was also found to be present in teleost soft tissues, predominantly in heart and kidney [15], with the expression of MGP in heart tissue mainly associated with two specific cell types, smooth muscle and endothelial cells [15].

We have recently developed specific polyclonal antibodies against BGP and MGP from the teleost fish *A. regius* [15] as well as against MGP purified from soupfin shark (*Galeorhinus galeus*) (our unpublished results). The purpose of the present work was to demonstrate the usefulness of these antibodies for studies of BGP/MGP accumulation and tissue distribution in marine and fresh water fish and amphibians. To achieve this goal we purified and characterized MGP and BGP from various fish species and from *Xenopus* and compared the ability of our antibodies to recognize these specific antigens.

Material and Methods

Extraction of MGP and BGP from Fish and Amphibian Tissues

Vertebra from *Sparus aurata*, *Solea senegalensis* and *Prionace glauca*, branchial arches from *Halobatrachus didactylus* and the entire skeleton of *Xenopus laevis* and *Danio rerio* were cleaned from adhering soft tissues and the Gla-containing proteins were extracted based on previously described procedures [14, 15, 17]. Briefly, the mineralized material was ground in a mortar with liquid nitrogen to less than 1 mm in diameter,

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extensively washed with 6 M guanidine HCl and water and dried with acetone. The resulting powder was demineralized with 10% formic acid, dialyzed (SpectraPor 3) against 50 mM HCl, freeze-dried, re-suspended in 6 M guanidine-HCl, 0.1 M Tris-HCl pH 9, 10 mM EDTA, further dialyzed (SpectraPor 3; Spectrum, Gardena, CA, USA) against 5 mM ammonium bicarbonate and freeze-dried [15]. *P. glauca* dialyzed extract showed a precipitate that was separated, dissolved in 50 mM HCl and stored at −80°C. Samples were then analyzed by SDS-PAGE as described in "Electrophoresis and Blotting."

For all the other preparations, the crude extracts were ultrafiltrated using a Centricon-10 with 10 kDa molecular weight cut-off (Amicon, Millipore, Bedford, MA, USA). The combined final filtrates containing BGP (lower than 10 kDa) were further desalted and concentrated using a Centricon-3 (3 kDa cut-off) (Amicon).

Both the filtrate and filter-retained samples were collected and their protein content was determined as described in "Protein Quantification." Proteins in each fraction were further analyzed by SDS-PAGE.

Protein Quantification

Total protein concentration in crude acid extracts was determined using the Commassie plus protein assay reagent (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. For the determination of protein concentration of purified BGP samples, the value of $E^{0.1\%\ 1\ cm} = 1.33$ at 280 nm [18] was used. For MGP concentration determination the value of $E^{0.1\%\ 1\ cm} = 1.0$ at 280 nm was used (P.A. Price, personal communication).

N-Terminal Protein Sequence Analysis

*Dr*BGP (*D. rerio* BGP) and *Hd*BGP (*H. didactylus* BGP) purified proteins were directly deposed on a polybrene-coated glass fiber filter. *Pg*MGP (10 μg) (*P. glauca* MGP) containing samples were loaded onto 4 lanes and fractionated on a 18% SDS-polyacrylamide gel, then transferred onto a PVDF membrane filter (Applied Biosystems, Foster City, CA, USA) and stained with Commassie Brilliant Blue in order to visualize protein bands. The band corresponding to *Pg*MGP (around 18 kDa) detected in each lane was cut for further identification by protein sequence analysis. Automatic Edman degradations were performed as described [13] using an Applied Biosystems Model 494 sequenator equipped with an on-line HPLC and employing the standard program supplied by the manufacturer. Phenylthiohidantoin (PTH) amino acid derivatives were separated by a 2.1 mm × 22 cm C-18 reverse-phase HPLC column (Applied Biosystems) and the gradient conditions were those recommended by Applied Biosystems.

Electrophoresis and Blotting

Total protein (20–30 μg) was dissolved in SDS sample buffer containing reducing agent (NuPage, Invitrogen, La Jolla, CA, USA), applied to a 12% or 4–12% gradient polyacrylamide precast gel containing 0.1% SDS (NuPage, Invitrogen) and fractionated at a constant 140 volts. The gels were stained either with 0.2% Commassie Brilliant Blue R-250 (C.I. 42660, Bio-Rad, Richmond, CA, USA), 10% trichloroacetic acid, 10% 5-sulfosalicylic acid or with a DBS-staining solution specific for Gla-containing proteins [(8.5 mM 4-diazobenzene sulfonic acid (DBS); Sigma, Spain; 6.4 mM $NaNO_2$ in 2 M acetate buffer, pH 4.6)] as described in [19]. Lysozyme (Sigma) and protease factor Xa (New England Biolabs) were used, respectively, as negative and positive controls for the DBS-staining method. Blotting onto nitrocellulose (Invitrogen) was performed for 1 h at constant 80 mA using a Bio-Rad Mini Trans-Blot Cell system (Bio-Rad) and a Bis-Tris transfer buffer (NuPage, Invitrogen). The membranes were blocked for 2 h with 5% (w/v) non-fat dried milk powder in TBST (15 mM NaCl, 10 mM Tris-HCl buffer, pH 8, 0.05% Tween 20; Blotto) and then incubated overnight with 0.04 μg/ml anti-*Ar*MGP or anti-*Ar*BGP affinity-purified antiserum in Blotto or with anti-*Gg*MGP polyclonal antibody diluted 1:100 in the same solution. Immunoreactive protein bands were detected using alkaline phosphatase-labeled goat anti-rabbit IgG antibody (Gibco-BRL, Paisley, UK) diluted 1:20,000 in TBST and visualized using NBT/BCIP substrate solution (Sigma) as described [20]. Negative controls consisted in the substitution of the primary antibody with phosphate-buffered saline (150 mM NaCl, 15 mM sodium phosphate buffer, pH 7.2; PBS) in the experiments with the purified antibody and with normal rabbit serum when using the non-purified antibody. Controls also included pre-incubation of the primary antibody for 1 h at room temperature with purified *Ar*MGP and *Ar*BGP purified protein (1:10 weight ratio).

Dot-blot Analysis

Purified protein samples were deposited in 1 μl aliquots (0.5 μg total protein) onto a nitrocellulose membrane (Invitrogen). Membranes were allowed to dry for 1 h and blocked for 2 h with 5% (w/v) dried milk powder in TBST. Incubation with anti-*Ar*MGP, anti-*Ar*BGP affinity-purified antibodies and anti-*Gg*MGP polyclonal antiserum and detection of immunoreactive protein spots was performed as described in "Electrophoresis and blotting."

ArBGP, ArMGP and GgMGP Antiserum

MGP and BGP were purified from formic acid extracts of *A. regius* branchial arches as described [15]. Rabbit polyclonal antibodies against MGP and BGP from *A. regius* were obtained from Strategic BioSolutions (Ramona, CA, USA) using the purified protein adsorbed to polyvinylpyrrolidone (PVP-40), as described [11, 15]. *Gg*MGP (MGP from *Galeorhinus galeus*) antiserum was raised against purified *Gg*MGP [21] using a described method [11].

Specificity of the antiserum was determined by Dot-blot and Western-blot immunoassays as described in "Electrophoresis and Blotting" using either the purified proteins (*Ar*MGP and *Ar*BGP) [15] or the characterized MGP obtained from a crude extract of *P. glauca* calcified cartilage.

Affinity Purification of ArBGP and ArMGP Antiserum

Purified protein samples [15] (5 mg of *Ar*BGP dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 and 1.2 mg of *Ar*MGP re-suspended in 0.2 M $NaHCO_3$, 4 M urea) were coupled to the CNBr-activated Sepharose 4B resin (Amersham-Pharmacia, Piscataway, NJ) overnight at 4°C. Adsorption of the *Ar*BGP and *Ar*MGP polyclonal antibodies was performed according to the manufacture's instructions. Bound antibodies were eluted with 100 mM glycine pH 2.5 followed with 100 mM triethylamine, pH 11.5 and samples (1 ml) were immediately neutralized by adding 100 μl of 1 M Tris-HCl, pH 7. Absorption of the effluent at 280 nm was monitored. The resulting peak fractions were dialyzed against phosphate-buffer saline and stored at −20°C.

Determination of BGP Theoretic Isoelectric Point

Isoelectric point of BGPs from *A. regius*, *S. aurata*, *D. rerio*, *X. laevis* and *B. taurus* were calculated using the Peptide Statistics Tool (PEPSTATS) from biotools (http://biotools.umassmed.edu). The complete mature sequence derived from cDNA was used in each case and values were calculated in the absence of either hydroxylation of proline or γ-carboxylation of glutamic acid residues.

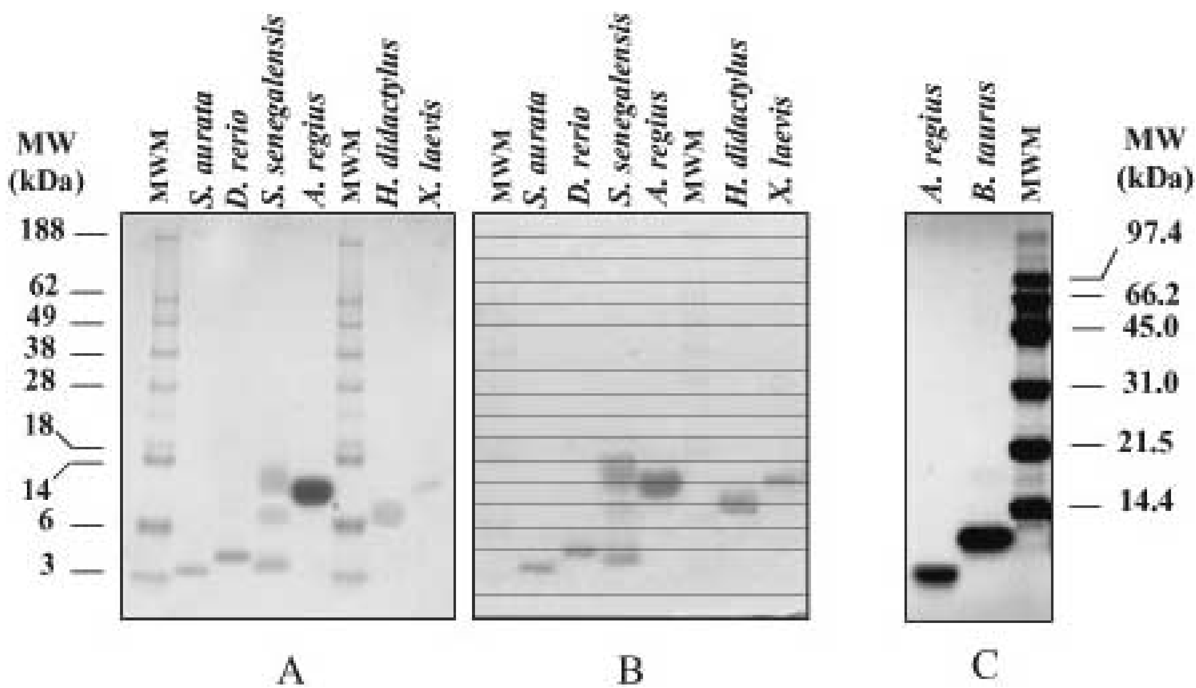


Fig. 1. Comparison of migration behavior on SDS-PAGE of Gla-containing BGPs from different teleosts (*S. aurata*, *D. rerio*, *S. senegalensis*, *H. didactylus*, *X. laevis* and *B. taurus*. (**A**) and (**B**) Each lane of 12% SDS-PAGE (Nupage, Invitrogen) was loaded with 10 µg of protein. One gel was stained using the CBB-staining method for total protein detection (**A**) and the other with DBS-staining method specific for Gla-containing proteins (**B**); MWM, SeeBlue pre-stained molecular weight markers (Invitrogen). (**C**) Protein 10 µg either *A. regius* or *B. taurus* purified BGP was loaded per lane on a 18% SDS-PAGE gel (Novex, Invitrogen) and stained with CBB; MWM, low range molecular weight markers from BioRad. Corresponding molecular weights (in kDa) are indicated on the margins of the figure.

Results

Identification of Gla-containing Proteins from Mineralized Tissues of Teleost and Cartilaginous Fishes and from Xenopus

BGP and MGP were separated from the acid extract of *S. aurata*, *H. didactylus*, *S. senegalensis*, *D. rerio* and *X. laevis* mineralized tissues by ultrafiltration, as described in Material and Methods. SDS-PAGE results depicted in Figure 1A demonstrate that most of the protein in BGP-containing filtrates from *S. aurata*, *D. rerio* and *H. didactylus* migrated as a single component with higher mobility than BGP purified from *A. regius* branchial arches, a migration behavior similar to *X. laevis* BGP (Fig. 1A). Only *S. senegalensis* BGP sample showed more than one band, and the reason for this discrepancy remains to be clarified. In addition, all fish and *Xenopus* BGPs showed a higher migration behavior than bovine BGP (Fig. 1A, C). Positive reaction (red-orange) obtained with the DBS-staining method (Fig. 1B) following SDS-PAGE clearly indicated that the purified proteins from all species were gamma-carboxylated.

Comparison of the migration behavior between fish (*S. aurata*, *S. senegalensis*, *A. regius*, *D. rerio*), *Xenopus* and bovine BGPs in non-denaturing conditions over a 18% Tris-Glycine native gel is depicted in Figure 2. The results confirm the purity of the fractions analyzed since in this type of gel all protein samples tested migrated essentially as a major band although a second less intense band is also seen that could be explained by the

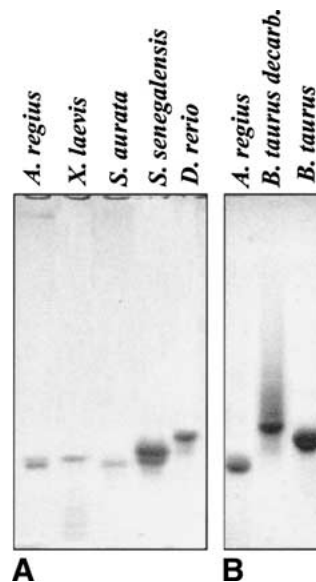


Fig. 2. Comparison of native electrophoresis migration behavior of BGPs from different fish species (*A. regius*, *S. senegalensis*, *S. aurata* and *D. rerio*), *X. laevis* and *B. taurus* applied to 18% Tris-glycine polyacrylamide gel (Novex, Invitrogen). Each lane was loaded with 10 µg of protein and the gel was stained using the CBB staining method. *B. taurus* decarb - *B. taurus* BGP decarboxylated (Poser and Price, 1979).

Table 1. Detection and characterization of BGP protein purified from bone and calcified cartilage acid extracts obtained from several teleost fishes (*A. regius*, *S. senegalensis*, *S. aurata*, *H. didactylus*, *D. rerio*) and *X. laevis*

	Specimen (tissue)	SDS-PAGE[a]		Immunodetection[b] (antibody)	N-terminal sequence[c]
		CBB	DBS		
BGP	*A. regius* (vertebra)	10 kDa	Positive	Western blot (anti-*Ar*BGP)	AAKELTLAQTE*SLRE*VCE* TNMACDEMADAQGIVAAY
	S. aurata (vertebra)	4 kDa	Positive	Western blot (anti-*Ar*BGP)	n.d.
	S. senegalensis† (vertebra)	4 kDa	Positive	Western blot (anti-*Ar*BGP)	n.d.
	D. rerio (skeleton)	5 kDa	Positive	Western blot (anti-*Ar*BGP)	AGTAXGDLTPFQLE*SLRE*VCE*
	H. didactylus (branchial arches)	8 kDa	Positive	Dot blot (anti-*Ar*BGP)	AAAELSLVQLE*SLRE*VCE*Q
	X. laevis (skeleton)	10 kDa	Positive	—*	n.d.

[a] The proteins were electrophoresed on a 18% SDS-PAGE (NuPage, Invitrogen) stained with CBB and DBS and the molecular weights were estimated by plotting the log versus relative migration distance using the pre-stained molecular weight markers (SeeBlue, Invitrogen) over a linear range
[b] Immunodetection of the proteins was performed by Western blot or Dot blot analysis using anti-*Ar*BGP affinity purified antibodies as primary antibody (0.1 μg) and alkaline phosphatase-labeled goat anti-rabbit IgG antibody with the NBT/BCIP substrate solution as secondary antibody
[c] N-terminal sequence was obtained as described in Materials and Methods section and is numbered from the first amino acid residue identified from sequence determination, E*, Gla residue. X, non-identified amino acid residue, (n.d.) not determined
† Besides this one band (3–6 kDa) there were 2 extra bands both coloring with CBB- and DBS-staining (SDS-PAGE, Figure 1) and recognized by the same antibody (Western blot, Figure 3)
* Affinity purified antibodies (anti-*Ar*BGP and anti-*Ar*MGP) as well as anti-*Gg*MGP polyclonal antiserum were tested and gave negative results

presence of a BGP with a different degree of carboxylation. In fact, the effect of carboxylation of BGP is quite clear once we compare, over a native gel, the migration of *B. taurus* BGP with a sample of decarboxylated protein (Fig. 2B) obtained using a described procedure [23].

N-terminal amino acid sequence analysis was obtained for *Dr*BGP and *Hd*BGP (Table 1) and their identity was confirmed based on the fact that the first 21 (*Dr*BGP) and 19 (*Hd*BGP) amino acid residues sequenced showed high homology with previously sequenced BGPs [15, 17]. The assignment of the three Gla residues in the N-terminal sequence (Table 1) was based on comparison with other BGP sequences [17] where these residues are highly conserved, and also on the fact that in the standard sequence analysis no PTH derivative could be identified at these positions. Western- blot analysis of the fish BGP-containing filtrates from *S. aurata*, *D. rerio*, *H. didactylus* and *S. senegalensis* using the anti-*Ar*BGP purified antibody (Fig. 3) also confirmed the identification of these bands as BGP.

Analysis by SDS-PAGE of the centricon 30 filter-retained contents of each sample followed by CBB and DBS staining permitted the identification of a Gla-containing protein in *X. laevis*, *S. aurata* and *S. senegalensis* sample extracts (Fig. 4) with a migration behavior (14–18 kDa) similar to that previously obtained for *Ar*MGP (Fig. 4). The *P. glauca* sample showed a clearly defined band (Fig. 4) that was sufficiently pure and abundant to obtain a N-terminal amino acid sequence (30 amino acids residues were obtained, see Table 2) and confirmed its identity to be *Pg*MGP based on its high homology with the previously sequenced

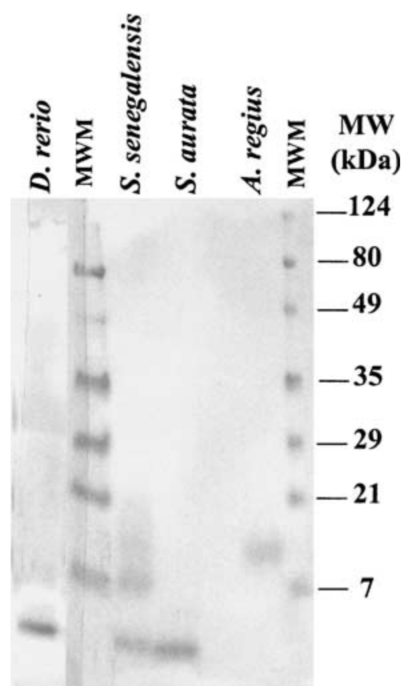


Fig. 3. Western blot analysis of BGPs from several teleost fish. 1 μg of each protein was electrophoresed on 18% SDS-PAGE (Novex, Invitrogen). Following electrophoresis, proteins were blotted onto nitrocellulose, and membranes were incubated with 0.04 μg/ml of anti-*Ar*BGP affinity purified antibody. Alkaline phosphatase-labeled goat anti-rabbit IgG wasused as secondary antibody and NBT/BCIP as substrate solution. MWM, pre-stained molecular weight markers from Bio-Rad. Corresponding molecular weight (in kDa) are indicated on the right margin of the figure.

soupfin shark MGP [21]. Western-blot analysis of the filter-retained content in each sample using either the

Table 2. Detection and characterization of MGP protein in crude or purified bone and calcified cartilage acid extracts obtained from several teleost fish (*A. regius*, *S. senegalensis*, *S. aurata*, *D. rerio*), *X. laevis* and blue shark (*P. glauca*)

	Specimen (tissue)	SDS-PAGE[a] CBB	SDS-PAGE[a] DBS	Immunodetection[b] (antibody)	N-terminal sequence[c]
MGP	*A. regius* (branchial arches)	14–18 kDa	Positive	Western blot (anti-*Ar*MGP)	YE*SHESXESAEDLFVPXQ XANSFMTPXRG
	D. rerio (skeleton)	14–18 kDa	Positive	Western blot (anti-*Ar*MGP)	n.d.
	S. senegalensis (vertebra)	14–18 kDa	Positive	Western blot (anti-*Ar*MGP)	n.d.
	S. aurata (vertebra)	14–18 kDa	Positive	Western blot (anti-*Ar*MGP)	n.d.
	X. laevis (skeleton)	14–18 kDa	Positive	Western blot (anti-*Gg*MGP)	n.d.
	P. glauca (vertebra, jaw, branchial arches)	14–18 kDa	Positive	Western blot (anti-*Gg*MGP)	DSSESNEIDDVLFLGRRDA NSFMKYPQLGN

[a] The proteins were electrophoresed on a 4–20% SDS-PAGE (NuPage, Invitrogen), stained with CBB and DBS and the relative molecular weight estimated by comparison with pre-stained molecular weight markers (SeeBlue, Invitrogen)
[b] Immunodetection of the protein was performed by Western blot or Dot blot analysis using the anti-*Ar* MGP purified antibodies (0.1 μg) or anti-*Gg* MGP polyclonal antiserum (1:100 dilution) as primary antibody and alkaline phosphatase-labeled goat anti-rabbit IgG antibody with the NBT/BCIP substrate solution as secondary antibody
[c] The N-terminal sequence presented in this table was obtained as described in Materials and Methods section and is numbered from the first amino acid residue obtained from sequence determination. X, non-identified amino acid residue. E*, Gla residue, (n.d.) not determined

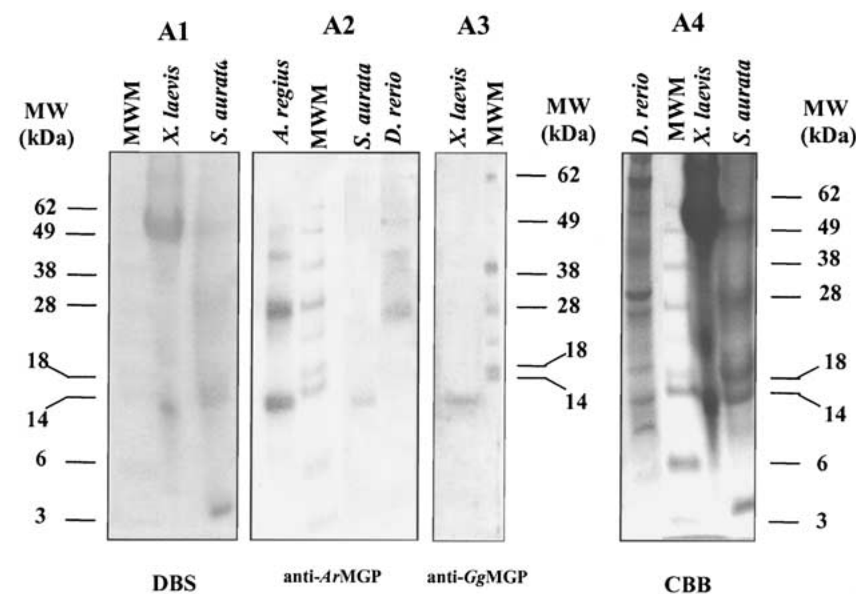
anti-*Ar*MGP purified antibody or the anti-*Gg*MGP polyclonal antiserum (Table 2) also confirmed the identification of these bands as MGP.

For the *S. aurata* and *S. senegalensis* retentate samples a single band with positive immunoreaction with anti-*Ar*MGP purified antibody was obtained (Fig. 4, A2, B2). These results together with the DBS-staining results performed on the same SDS-PAGE that also gave positive coloration for a band with the same migration behavior (Fig. 4, A1, B1) confirmed that the protein was γ-carboxylated and that there was no evidence of MGP aggregation. The presence of a second band in the same filter-retained extract (and a third band in the case of *S. senegalensis* sample) with a positive coloration by the DBS-staining but with a higher migration behavior suggested that BGP was present in both *S. aurata* and *S. senegalensis* filter-retained samples (Fig. 4, A1, B1). Western blot using anti-*Ar*BGP purified antibody as a control in these samples, gave positive immunoreaction for BGP confirming the hypothesis of the presence of BGP in *S. aurata* and *S. senegalensis* filter-retained samples (results not shown).

Western-blot analysis of *X. laevis* filter-retained content identified a band around 14–18 kDa with positive immunoreaction with anti-*Gg*MGP polyclonal antiserum that also gave positive coloration with DBS-staining performed on the same SDS-PAGE, confirming that the protein was γ-carboxylated (Fig. 4, A3, A1). In the case of *D. rerio*, the results obtained by Western blot showed three bands that positively immunoreacted with anti-*Ar*MGP antibody with a pattern of migration behavior similar to *Ar*MGP (Fig. 4, A2). The absence of any detectable band around 14–18 kDa is possibly due to protein aggregation during the ultrafiltration procedure performed using 5 mM ammonium bicarbonate. This phenomenon is characteristic of MGP especially when stored in non-denaturing buffers as previously referred to [23, 24]. A comparable result was also observed, although to a less extent, for the *Ar*MGP sample (Fig. 4, A2). However, and in agreement with results obtained for *A. regius* (results not shown), no coloration by DBS-staining was observed for this *Dr*MGP sample (result not shown) probably because of the protein aggregation limiting the exposure of the Gla residues to the staining, an essential feature to obtain a positive result with this staining procedure.

Given the complexity of the protein mixture content in *X. laevis*, *S. senegalensis*, *S. aurata* and *D. rerio* filter-retained samples, the low efficiency obtained on the separation over a 4–12% gradient SDS-PAGE (Fig. 4) and also the low MGP content and the aggregation phenomenon, it was not possible to obtain a clear N-terminal amino acid sequence for these samples.

*Pg*MGP sample analysis by Western blot with anti-*Gg*MGP polyclonal antiserum showed a positive immunoreaction for the 14 kDa band but a second band was also identified in the precipitant fraction with a higher migration behavior (around 6 kDa) (Fig. 4, C1, C2). This band probably represents fragments of MGP,



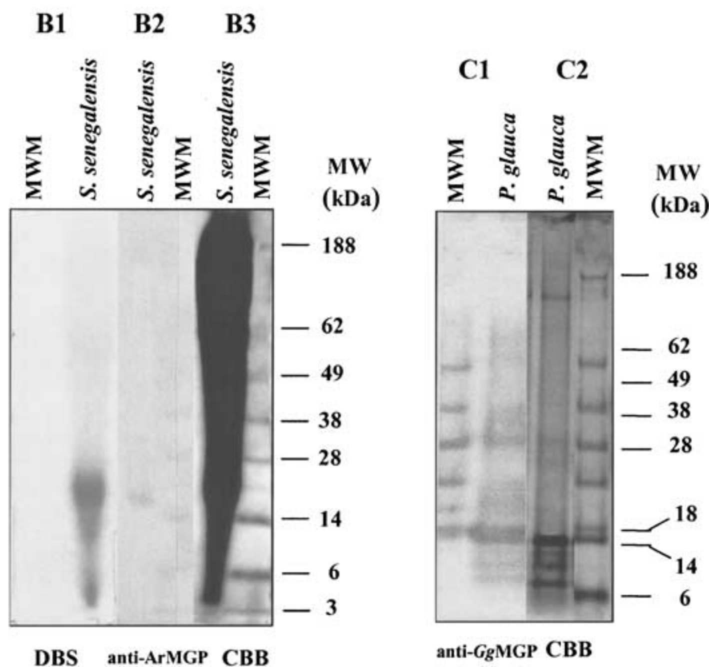
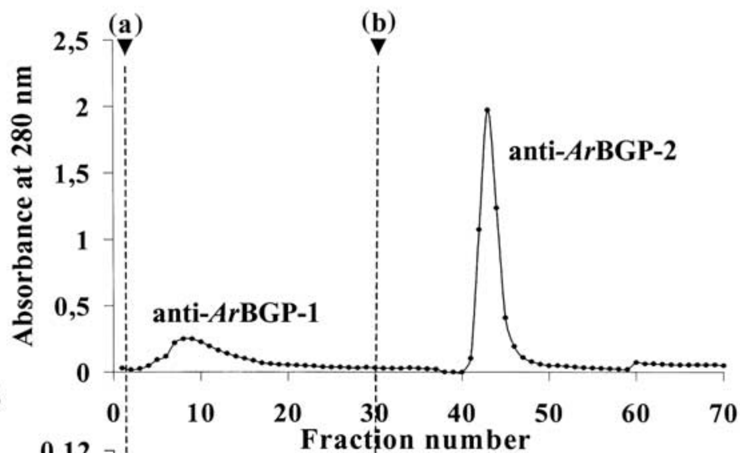


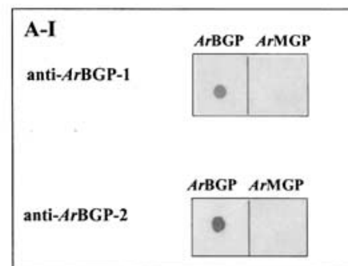
Fig. 4. SDS-PAGE and Western-blot analysis of several teleost fish (*S. aurata*, *S. senegalensis*, *D. rerio*, *A. regius*), *X. laevis* and *P. glauca* dialyzed acid extracts containing MGP. Electrophoresis was performed using 4–12% SDS-PAGE gels (Nupage, Invitrogen). Each lane was loaded with 30–50 μg of total protein. CBB: Gels were stained using the CBB staining method for total protein detection (A4, B3, C2). DBS: Gels were stained using the DBS staining method specific for Gla residues (A1, B1). anti-*Ar*MGP and anti-*Gg*MGP: for Western blot analysis, a 1:10 dilution of the samples was used to load the gel. Following electrophoresis, proteins were blotted onto nitrocellulose, and the membranes were incubated either with 0.04 μg/ml of anti-*Ar*MGP purified antibody (A2, B2) or 1:100 dilution of anti-*Gg*MGP polyclonal antiserum (A3, C1). Alkaline phosphatase-labeled goat anti-rabbit IgG was used as secondary antibody and NBT/BCIP as substrate solution as described in the Materials and Methods. MWM, SeeBlue pre-stained molecular weight markers (Invitrogen).

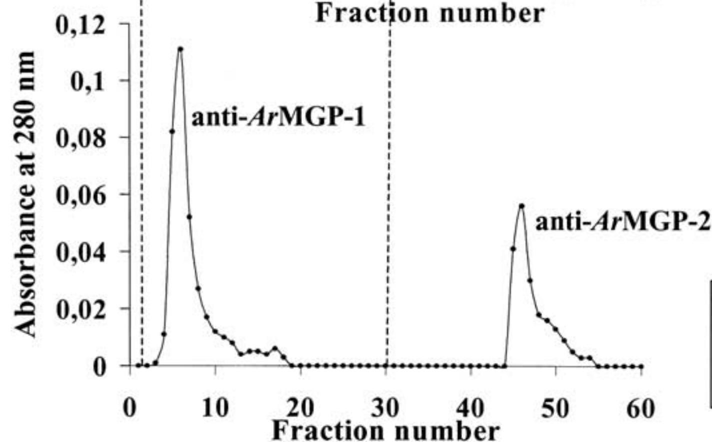
a result previously obtained in *Ar*MGP purification from teleost fish-calcified cartilage [15]. Western blot analysis of the insoluble and the soluble dialyzed fraction using the anti-*Ar*BGP antibodies gave negative results (results not shown).

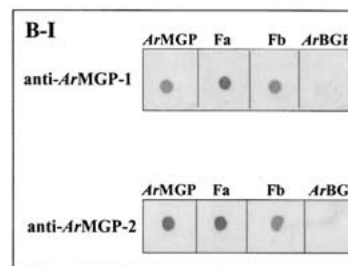
Antiserum Purification and Characterization

For use in immunohistochemistry and in the analysis of cell protein extracts, *Ar*BGP and *Ar*MGP polyclonal antisera were affinity-purified, as described in Materials and Methods, using *Ar*BGP and *Ar*MGP purified from *A. regius* branchial arches [15]. As shown in Figures 5A and 5B, the affinity purification elution profile of both antisera permitted the separation of two different antibody entities, anti-*Ar*BGP-1 and anti-*Ar*BGP-2 in *Ar*BGP polyclonal antiserum (5A) and anti-*Ar*MGP-1 and anti-*Ar*MGP-2 in *Ar*MGP polyclonal antiserum (5B). Comparing the relative amount of the elution peaks obtained, anti-*Ar*BGP-2 (5A) and anti-*Ar*MGP-1 (5B) purified antibodies seemed to be the most abundant in the unpurified anti-*Ar*BGP and anti-*Ar*MGP antiserum, respectively. Dot blot analysis









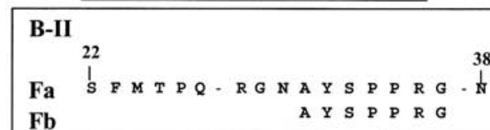


Fig. 5. Affinity purification of anti-*Ar*BGP (**A**) and anti-*Ar*MGP (**B**) polyclonal antiserum using CNBr-activated Sepharose 4B resin coupled with the corresponding antigen. Bound antibodies were eluted with approximately 100 mM glycine pH 2.5 (**a**), followed with 100 mM triethylamine, pH 11.5 (**b**). Fractions of approximately 1 ml were collected every 5 minutes. Elution profile was obtained by measuring absorbance of effluent fractions at 280 nm. Peak fractions were collected and dialyzed with phosphate buffer saline (PBS). Immunoreaction of 0.1 µg of *Ar*MGP, *Ar*BGP or polypeptide *Ar*MGP fragments (Fa and Fb, B-II) upon treatment with 0.04 µg/ml of anti-*Ar*BGP purified antibodies (A-I) and anti-*Ar*MGP purified antibodies (B-I) obtained by Dot-blot analysis, as described in Materials and Methods.

using different anti-*Ar*BGP purified antibody dilutions against various concentrations of the pure antigen showed that anti-*Ar*BGP-1 and -2 recognized the BGP antigen with the same degree of sensitivity (results not shown).

Characterization of anti-*Ar*MGP-purified antibodies by Dot-blot analysis also showed that anti-*Ar*MGP-1 and -2 recognized both *Ar*MGP antigen and the purified *Ar*MGP fragments [15] with the same degree of sensitivity, as tested by Dot blot analysis (Fig. 5B-I, B-II). Relative specificity of these antibodies was further tested using different antibody dilutions against various concentrations of purified MGP and polypeptide fragments and shown to be both equally specific for the fragments and the mature protein (results not shown).

Our results also indicate that the anti-*Ar*MGP and anti-*Ar*BGP purified antibodies were 100 times more sensitive than the unpurified antiserum since a dilution of 1:10,000 (corresponding to the same total protein concentration of originally unpurified antiserum) could detect 10 ng of purified antigen (results not shown).

Immunodetection of BGP and MGP Proteins from Different Teleost Fish, X. laevis and P. glauca Using ArBGP, ArMGP and GgMGP Antibodies

Specificity of both the affinity-purified anti-*Ar*BGP and anti-*Ar*MGP and the non-purified anti-*Gg*MGP antibodies was confirmed by Western blot analysis. The specificity was further tested by comparing its reaction with BGP and MGP proteins extracted from several teleost, a cartilaginous fish and *Xenopus*.

Recognition of *S. aurata*, *S. senegalensis and D. rerio* BGPs by anti-*Ar*BGP purified antibodies was assessed

Table 3. Immunodetection results for MGP and BGP extracted from several teleost fishes (*A. regius*, *S. aurata*, *S. senegalensis*, *D. rerio*), *X. laevis* and *P. glauca* using the affinity purified anti-*Ar*MGP (0.1 µg) and anti-*Ar*BGP antibodies (0.5 µg) and the anti-*Gg*MGP polyclonal antiserum (1:100 dilution). Relative specificity was deduced from Western blot and Dot blot analysis (as described in Materials and Methods) using the same concentration of antigen and primary antibody

Antibody	Antigen	Species	Relative specificity
		A. regius	+ + +
anti-*Ar*BGP	BGP	*S. aurata*	+ +
		S. senegalensis	+ +
		H. didactylus	+ +
		D. rerio	+ +
		X. laevis	–
anti-*Ar*MGP	MGP	*A. regius*	+ + +
		S. aurata	+ +
		S. senegalensis	+ +
		P. glauca	+
		D. rerio	+ +
		X. laevis	–
anti-*Gg*MGP	MGP	*P. glauca*	+ + +
		X. laevis	+ +
		A. regius	+ +

by Western blot (Fig. 3) using the proteins purified from acid extracts of calcified tissues (Table 1). A positive reaction with anti-*Ar*BGP antibodies was seen by Dot blot assay with *H. didactylus* BGP while no positive reaction was observed for *Xl*BGP (Table 1).

Positive immunoreaction of *S. aurata*, *S. senegalensis* and *D. rerio* MGPs was seen by Western blot analysis using the anti-*Ar*MGP purified antibodies as depicted in Figure 4. However, this antibody did not recognize *X. laevis* MGP whereas *P. glauca* MGP was recognized but with a lower specificity than teleost fish MGPs (Table 3). However, both *P. glauca* and *X. laevis* MGPs were immunodetected by Western blot using anti-*Gg*MGP polyclonal antiserum (Table 3).

Discussion

Based on previous results obtained in higher vertebrates (*B. taurus*) [25], in *X. laevis* and the teleost fishes *Xiphias gladius*, *S. aurata* and *A. regius* [15, 17, 26], we were able to purify and characterize BGP from the vertebra and jaw of several other marine teleost fishes (*S. senegalensis* and *H. didactylus*), and from the entire mineralized skeleton of a fresh water teleost (*D. rerio*). In contrast with mammals, two major histological types of bone can be found in teleost fish (cellular and acellular bone) [27, 29]. Despite these structural differences, our results indicate that in fish, as in mammals [26], fish bone-like mineralized tissue is the major site of accumulation for BGP [14, 15, 17] supporting the hypothesis that BGP function is likely to be associated with bone in all vertebrates. Comparison of *A. regius* BGP SDS-PAGE migration profile with those from other teleosts (*S. senegalensis*, *S. aurata*, *H. didactylus* and *D. rerio*), as well as from *X. laevis* and *B. taurus*, showed clear differences. While *X. laevis*, *H. didactylus* and *A. regius* BGPs had a similar behavior and migrated more like the *B. taurus* protein, the *S. aurata* and *D. rerio* BGPs migrated as smaller entities (Fig. 1). This result cannot be explained solely by the known molecular weight difference between the proteins, or by the differences in gamma-carboxylation, since the results of N-terminal sequence obtained (*A. regius* [15], *H. didactylus* and *D. rerio*, (this study), *S. aurata* and *X. laevis* [17], and *B. taurus* [29]) showed that BGP was always fully gamma-carboxylated at each of the three Gla residues. Comparison between calculated isoelectric points (pI) (Table 4) also does not reflect the anomalous migration behavior in SDS-PAGE since they all have low pIs. In contrast, the relative migration behavior of these BGPs on native polyacrylamide gels (Fig. 2) correlates better with the differences observed between their calculated pIs (Table 4), suggesting the presence of different levels of gamma-carboxylation.

Comparing the gel filtration purification profiles for BGP from different fishes (swordfish [26], *S. aurata* [17], *L. macrochirus* [16] and *A. regius* [15]) and considering the different levels of purity achieved in each reported experiment using similar techniques, we conclude that the BGP content in bone-like mineralized tissues seems to be dependent not only on the origin of the tissue but also on the fish species and stage of development. Different contents of BGP in vertebra may reflect not only the cellular versus acellular nature of the bone (depending on the species analyzed) but also on the requirement for this protein, which may vary depending on growth stage in teleosts.

This study reports for the first time the identification of MGP in acid extracts of teleosts vertebra. The identification of MGP in the teleosts *S. senegalensis*, *S. aurata* and *D. rerio* suggests that the level of MGP accumulation in teleost fishes mineralized tissues is also probably dependent on the stage of development since previous experiments failed to purify MGP from teleost fish vertebra acid extracts [15, 17]. This evidence is further supported by our previous immunohistochemistry results where we found that MGP can accumulate in *A. regius* neural arch vertebra in regions counterstaining with Alcian Blue (stains positive for mucopolyssacarides) [15]. The full characterization of this extracellular matrix by *in situ* hybridization and immunohistochemistry will be the subject of future studies once additional specific molecular markers are available for this fish, a work presently ongoing in our laboratory. Differences between the levels of MGP accumulation in vertebra of young and adult fishes may also

Table 4. Comparison of descriptive information based on BGP amino acid sequences of *B. taurus*, *X. laevis*, *A. regius*, *S. aurata* and *D. rerio* obtained using Peptide Statistics Tool (PEPSTATS) from biotools (http://biotools.umassmed.edu)[a]

		B. taurus	*X. laevis*	*A. regius*	*S. aurata*	*D. rerio**
	Molecular weight (Da)	5704.33	5359.79	4868.49	4950.63	5209.89
	Isoelectric point (pI)	4.21	4.08	3.76	3.87	4.10
	Number of residues	49	49	45	45	48
Types of residues	Aliphatic	8	8	7	10	8
	Aromatic	9	6	4	5	6
	Non-polar	31	28	28	29	30
	Polar	18	21	17	16	18
	Charged	16	12	9	8	9
	Basic	6	4	2	2	3
	Acidic	10	8	7	6	6

Sequence GenBank accession numbers are: AF459030 for *Argyrosomus regius*; AF048703 for *Sparus aurata* (seabream); AF055576 for *Xenopus laevis*; X53699 for bovine (*Bos taurus*); AY078413.1 for *Danio rerio*
* The complete mature sequence derived from nucleotic analysis was used for this comparison obtained from AY078413.1 (Gavaia, Simes et al., 2003 submitted)
[a] Values were calculated for BGP in the absence of hydroxyproline and γ-carboxylation of glutamic acid residues

reflect changes in the degree of mineralization found throughout development and in adult life and could be related to variable MGP synthesis rates at different periods of life or different requirement for MGP as the fish grows. These results are in contrast with data obtained in mammals in which it was shown that MGP accumulates at similar levels in bone of fetus or adult rats [11]. The presence in teleost fish MGPs of an extended C-terminus enriched in basic amino acids also found in shark [15, 21] and never found in mammalian MGPs, could be one of the reasons for the apparent higher affinity of the MGP for the mucopolisacaride-rich extracellular matrix found in branchial arches calcified cartilage and vertebra of fish.

Following the purification of MGP and BGP from *A. regius* mineralized tissues, the generation of specific polyclonal antibodies was successfully accomplished, as seen when tested for specificity and sensitivity for each specific antigen by Western blot analysis. The results obtained clearly showed that the anti-*Ar*MGP polyclonal antiserum only recognized fish MGP and not fish BGP and that anti-*Ar*BGP polyclonal antiserum was also specific for BGP [15].

To increase antibody specificity for use in immunohistochemistry studies, both polyclonal antisera were affinity-purified. Analysis of their purification profile suggested the presence of different antigen recognition sites in these antibodies, resulting in the purification of two separate peaks, each one in different elution conditions (acid and basic) (Fig. 5A, B). Both antibody-containing fractions resulted in comparable results when tested for immunoreactivity at different dilutions against the purified mature proteins. In the case of anti-*Ar*MGP antibodies, immunoreactivity against the purified MGP polypeptide fragments was also tested and both purified anti-*Ar*MGP antibody entities gave comparable results (Fig. 5B-I, B-II). Based on these results and since the two *Ar*MGP fragments comprised most of the mature protein (fragment Fa extended from residue 22 to the C-terminal residue, fragment Fb from residue 31 to the C-terminal residue) the possibility that the two antibodies recognize different epitopes cannot be inferred.

Immunodetection results obtained by Western blot using the affinity purified anti-*Ar*MGP and anti-*Ar*BGP antibodies showed that anti-*Ar*MGP specifically identified MGPs extracted from *S. senegalensis*, *S. aurata*, *D. rerio* and to a lower extent from *P. glauca* but did not immunoreact with *X. laevis* MGP (Table 3), a result that can be explained by the higher degree of sequence homology of the *X. laevis* sequence with MGPs from mammals and birds than with *A. regius* MGP [15]. Interestingly, the *X. laevis* MGP was found to immunoreact with another MGP polyclonal antiserum previously developed against shark MGP (anti-*Gg*MGP), a result that is not surprising given the features that shark and *X. laevis* MGPs have in common, namely the presence of an aspartic acid at position 2 and not a Gla residue which is seen in all other MGP sequences including *A. regius* [15]. However, this reaction was not as strong as was seen with MGP purified from a different shark (*Prionace glauca*) as expected.

Immunoreactivity of BGPs isolated from *S. senegalensis*, *S. aurata*, *D. rerio* and *H. didactylus* was also tested by Western blot and the results clearly showed that the purified anti-*Ar*BGP polyclonal antibodies were able to specifically recognize BGP purified from each of these different teleosts. As seen for MGP, the purified *Ar*BGP polyclonal antibody did not recognize BGP extracted from *Xenopus* bone (*Xl*BGP) (Table 3). This result has some consistency after comparison with the presently known fish BGP sequences [15, 30] since amphibian BGP is more closely related to bird and mammalian BGPs than to the fish proteins. The most notable

features are the conserved N-terminal insertion in bird, mammalian and *Xenopus* that is absent in teleosts and a homologous C-terminal in all *Xenopus*, bird and mammalian BGPs that is not conserved in fish BGPs.

The validation of these important biochemical tools in the recognition of either BGP or MGP antigen from different teleosts species, as well as from *X. laevis* and *P. glauca*, which are currently being studied in our laboratory, should be useful to further understand the deposition, distribution and developmental appearance of these proteins in non-mammalian vertebrates, thus providing further insight into its function throughout vertebrate evolution. The purified antibodies can now allow the establishment of specific immunoassays to adequately measure circulating levels of fish BGP and MGP, as already seen in the mammalian system. Another potential use of these tools may be to simplify purification procedures since they can be used to affinity purify these Gla-containing proteins from bone and calcified cartilage demineralized extracts.

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