BIOCHEMICAL CHARACTERIZATION OF THE SERUM FETUIN-MINERAL COMPLEX

Paul A. Price *, Thao Minh Thi Nguyen, and Matthew K. Williamson
Division of Biology, University of California, San Diego
La Jolla, Ca. 92093-0368

Running title: Characterization of the Serum Fetuin-mineral complex.

* Corresponding Author: Dr. Paul A. Price
Division of Biology, 0368
University of California, San Diego
La Jolla, Ca 92093-0368
Phone: 858-534-2120
Fax: 858-534-1492
email: pprice@ucsd.edu

This work was supported in part by US Public Health Service Grant AR25921.

SUMMARY

The present study was carried out to characterize the fetuin-mineral complex (FMC), a high molecular weight complex of calcium phosphate mineral and the proteins fetuin and matrix Gla protein (MGP) that was initially discovered in serum of rats treated with etidronate, and appears to play a critical role in inhibiting calcification *in vivo*. Fetuin purified from the FMC contains 3.3 moles of protein-bound phosphate. There is 1.3mg FMC per ml serum 6h after etidronate injection, and the FMC is 46% fetuin and 53% mineral by weight. Formation of the FMC in the first 6h after etidronate injection does not increase serum fetuin in spite of the fact that 50% of serum fetuin is associated with the FMC, and clearance of the FMC in the 9 to 24h interval lowers total serum fetuin by 50%. These observations suggest that the fetuin component of the FMC is derived from fetuin initially in serum, and that clearance of the FMC removes the associated fetuin from circulation. One additional protein was consistently present in all preparations of the FMC, secreted phosphoprotein 24 (spp24). This 24kDa protein is similar in domain structure to fetuin and, like fetuin and MGP, contains several residues of phosphoserine and accumulates in bone. Exogenous spp24 associated strongly with the FMC when added to serum containing it. These observations suggest that spp24 may, like fetuin and MGP, play a role in inhibiting calcification.

INTRODUCTION

The present experiments are a continuation of our investigations into the mechanisms by which proteins interact specifically with mineral *in vivo* to prevent the calcification of arteries and other soft tissues. In a previous study (1), we described the discovery of a complex of calcium, phosphate, fetuin, and matrix Gla protein in the serum of rats treated with the bone active bisphosphonate etidronate, and showed that the appearance of this complex in serum correlates with the inhibition of bone mineralization by etidronate. The fetuin-mineral complex reaches maximal levels 6 to 9h hours following etidronate injection and causes a 4-fold increase in total serum calcium without causing any increase in ionic calcium levels.

The proteins associated with the fetuin-mineral complex, fetuin and matrix Gla protein (MGP[1]), have both been shown to function as potent inhibitors of calcification *in vitro*. Fetuin is a 59 kDa protein that consists of 2 N-terminal cystatin domains and a smaller C-terminal domain. Fetuin is synthesized by the liver and secreted into blood, where it is found at a concentration of about 1mg/ml in the rat (2). Previous studies have demonstrated that fetuin is the major calcification inhibitor found in serum (3,4). Fetuin is also one of the most abundant non-collagenous proteins found in mammalian bone (5-10), with a concentration of about 1mg fetuin per g bone in rat (9). MGP is a small, 10 kDa vitamin K-dependent protein (11) which is secreted by a wide variety of cell types, including vascular smooth muscle cells, and is found in bone at a concentration of 0.2mg/g in the rat (12). Genetic and biochemical studies have convincingly demonstrated that impaired MGP function causes extensive calcification of the elastic lamellae in the artery media of mice (13), rats (14), and humans (15).

Our working hypothesis is that the calcification inhibitory properties of fetuin and MGP may be related to their ability to form complexes with nascent mineral nuclei that prevent the

growth of the mineral and target it for clearance from blood. This hypothesis is supported by the observation that formation of the fetuin mineral complex prevents the growth, aggregation, and precipitation of the mineral component (1), and by the observation that the fetuin-mineral complex is removed from blood in the 9 to 24h interval following etidronate injection (1).

The objectives of the present investigations were to better characterize the composition of the fetuin-mineral complex, to identify additional protein components in the complex, and to determine the impact of the generation and clearance of the complex on total serum levels of fetuin. In the course of these studies we have developed the first method for isolating the native, phosphorylated form of fetuin from rat serum and the first immunoassay for the determination of rat fetuin in serum and in other biological specimens.

EXPERIMENTAL PROCEDURES

Materials. Albino male rats (Sprague-Dawley derived) were purchased from Simonsen labs (Gilroy, CA). Etidronate was a gift from Proctor and Gamble (Cincinnati, OH). Sephacryl S-300 HR gel filtration media was purchased from Pharmacia (Piscataway, NJ). Warfarin and human serum amyloid P component (Amyloid P) were purchased from Sigma (St. Louis, MO), and human platelet factor 4 (PF4) was purchased from Calbiochem (La Jolla, CA). NuPAGE Bis-Tris 4-12% polyacrylamide gradient gels were purchased from Invitrogen Corp.(Carlsbad, CA). MGP and secreted phosphoprotein 24 (spp24) were purified from bovine bone as described (16,17). All other reagents used were reagent grade or better.

Purification of rat fetuin. In a typical experiment, 6 forty-day-old male rats were exsanguinated 6h after receiving a subcutaneous injection of 32mg etidronate / 100g body weight. Blood was allowed to clot for 30 min at room temperature and serum was collected by centrifugation at 1,400 X g for 10 minutes, pooled, and frozen in sixteen 1ml aliquots at -70˚C until use. The 1ml aliquots were centrifuged for 2h at 16,000 X g. The supernatants were then removed and the pellets were rinsed briefly with 1ml of ice cold 0.15M NaCl, resuspended in 0.5ml of HEPES buffer (20mM HEPES buffer pH 7.4 containing 1mM $CaCl_2$, 2mM Na_2HPO_4, 0.02% Azide, and 0.15M NaCl) and centrifuged for 30min at 16,000 X g. After removal of the supernatants, the pellets were dissolved serially in a 1ml volume of 60 mM EDTA pH 7.5 and applied directly to a 2 X 140 cm column of Sephacryl S300HR equilibrated with 5 mM NH_4HCO_3 at room temperature. Fractions of 3ml were collected and the absorbance at 220nm was determined; fetuin protein concentrations were estimated based on the assumption that a 1mg/ml solution of fetuin has an absorbance of 10 at 220nm. The ratio of absorbance at 220nm to absorbance at 280nm for purified rat fetuin is 32.4. Fetuin-containing fractions were either

used directly or concentrated to dryness by speed vac and then resuspended in the desired volume of buffer.

Analytical Methods. For determination of MGP, samples were assayed in triplicate using radioimmunoassay procedures previously described (12,13). Calcium levels in serum and other samples were determined colorimetrically using cresolphthalein complexone (Sigma) and phosphate levels in serum and other samples were determined colorimetrically as described (18). Protein sequencing was carried out on bands transferred to PVDF membranes using a Procise 494 Sequencer (ABI division, PE Biosystems, Foster City, CA). The phosphorylation of fetuin samples was determined using a modification of the Malachite Green procedure. In a typical experiment, 60µg of protein in 150µl of water was combined with 20µl of 8M NaOH and hydrolyzed for 30 min at 100˚C. The reaction was stopped by the addition of 30 µl of 10M HCl. Fifty microliter aliquots of the hydrolysate or of phosphate standards in 1.5M HCl were placed in wells of a 96 well microtiter plate and 100µl of Malachite Green reagent (Biomed Research Laboratories, Plymouth Meeting, PA) was added to each well and allowed to react for 90 min at room temperature. Results were obtained by measurement of absorbance at 600 nm. The moles fetuin in a sample were calculated from the absorbance at 220nm using the assumptions that the protein portion of fetuin has a molecular weight of 36,000 and that the protein weight can be estimated from the relation that a 1mg/ml solution of fetuin has an absorbance of 10 at 220nm.

Radioimmunoassay for rat fetuin. Rabbits were immunized against purified rat fetuin by Strategic Biosolutions (Newark, DE), and the antiserum was used at a final 1:8000 dilution. The radioimmunoassay diluent, sample volumes, and procedures were identical to those used in the MGP radioimmunoassay (12), with the exception that the iodinated fetuin and the sample (serum or extract) were added to antibody at the same time. Fetuin was iodinated by incubating

10μg of purified rat fetuin with 0.5mCi of ^{125}I and 4μg of iodogen (Pierce Chemical Co.) in 150 μl of 100mM phosphate buffer pH7.4 for 1h at room temperature.

Maintenance of animals. Rats were fed rodent diet 5001 (Purina Mills Inc., St. Louis, MO), a diet that is 0.67% phosphorus and 0.95% calcium by weight. Etidronate was dissolved in water, titrated to pH 7.4 with NaOH, and administered subcutaneously to forty-day-old male rats at a dose of 8 or 32mg per 100g body weight. To determine the effect of warfarin on the generation of the serum protein-mineral complex, rats received subcutaneous injections of 15.4mg warfarin/100g body weight 2h prior to etidronate injection. For plasma analyses, blood was drawn into a heparin-containing syringe to achieve a final heparin concentration of 75μg/ml and plasma was collected by centrifugation at 1,400 X g for 10 minutes in a clinical centrifuge. For serum, blood was allowed to clot for 30min at room temperature and serum was collected by centrifugation at 1,400 X g for 10 minutes. The UCSD Animal Subjects Committee approved all animal experiments.

Biochemical characterization of the fetuin mineral complex. To determine the composition of the fetuin-mineral complex at the 32mg /100g body weight dose of etidronate, eight rats received etidronate at t = 0. Four of these rats were exsanguinated 6h later and 4 rats were exsanguinated 24h later. Three 200μl aliquots of pooled serum obtained at the two time points were centrifuged at 16,000 X g for 2h to sediment the fetuin-mineral complex. After removal of the supernatant, pellets were washed briefly with 200μl of ice cold 0.15M NaCl and then dissolved overnight in 100μl of 0.15M HCl. Each solution was analyzed for calcium, phosphate, fetuin, and MGP; the numbers shown in Figure 4 and Table 2 are the average of the three individual determinations on each serum pool.

To determine the composition of the fetuin-mineral complex at the 8mg /100g body weight dose of etidronate, four rats received etidronate at t = 0 and were exsanguinated 6h later. To sediment the fetuin-mineral complex, three 175µl aliquots of pooled serum were placed in polyallomer centrifuge tubes and centrifuged for 1h in a Beckman airfuge operated at 27psi (approximately 164,000 X g)**.** After removal of the supernatants, pellets were washed briefly with 175µl of ice cold 0.15M NaCl and then dissolved overnight in 70µl of 0.15M HCl. Each solution was analyzed for calcium, phosphate, fetuin, and MGP; the numbers shown in Table 2 are the average of the three individual determinations.

Identification of additional constituents of the fetuin-mineral complex. To identify additional proteins in the fetuin-mineral complex, rats were exsanguinated 6h after etidronate injection. For samples from animals treated with 32mg etidronate/100g body weight, 1ml aliquots of pooled serum or plasma were centrifuged at 16,000 X g for 2h to sediment the fetuin-mineral complex. After removal of the supernatant, pellets were washed briefly with 0.5 ml of ice cold 0.15M NaCl and resuspended in 0.5ml of HEPES buffer. Aliquots were again centrifuged for 30min at 16,000 X g and the supernatants were removed. For samples from animals treated with 8mg etidronate/100g body weight, 175µl aliquots of pooled serum or plasma were centrifuged at 164,000 X g for 1h to sediment the fetuin-mineral complex. After removal of the supernatants, pellets were washed briefly with 175µl of ice cold 0.15M NaCl and then resuspended in 40µl of HEPES buffer. Aliquots were again centrifuged for 1h at 164,000 X g and the supernatants were removed. The final pellets from the 8 and 32mg doses were dissolved in SDS gel loading buffer containing 60 mM EDTA pH 7.4 and electrophoresed on 4 to 12 % polyacrylamide gels. Proteins were either stained with Coomassie or electrophoretically transferred to a PVDF membrane for N-terminal protein sequencing.

Studies were also carried out to see if the addition of purified MGP, platelet factor 4, secreted phosphoprotein 24, or serum amyloid P component to serum from etidronate-treated rats would increase the level of each in the fetuin-mineral complex. In a typical experiment, serum was obtained from rats 6h after injection of 32mg etidronate/100g, and 200µl of this serum was placed into each of three tubes. One tube received 5µg of purified platelet factor 4, the second received no addition, and the third received 5µg of platelet factor 4 and 50 mM EDTA. Each tube was centrifuged for 2h at 16,000 X g. The supernatants were then removed and the tubes were rinsed briefly with 200µl of ice cold 0.15M NaCl. The three pellets were then dissolved in SDS gel loading buffer containing 60 mM EDTA, and 50% of the dissolved pellet was electrophoresed on a 4 to 12% polyacrylamide gel; the control lane contained 2.5µg of platelet factor 4 alone. This procedure was repeated to test the possible incorporation of 20µg of secreted phosphoprotein 24 and 6µg of purified human serum amyloid P component into the fetuin-mineral complex. For MGP, 100µg of the protein was added to 200µl of serum and only 10% of the dissolved pellet was loaded onto the gel.

RESULTS

Purification of fetuin from the serum of etidronate-treated rats. The native form of rat fetuin was purified from the serum fetuin-mineral complex for use in immunizations and immunoassays. The first step in purification was sedimentation of the fetuin-mineral complex by centrifugation of serum from etidronate-treated rats. In agreement with previous studies (1), the resulting pellets were primarily fetuin with significant contamination by serum albumin (**Figure 1**). To remove albumin and other non-specifically associated contaminants, the pellets were resuspended in HEPES buffer and the fetuin-mineral complex was again sedimented by centrifugation. As seen in lane 3 of **Figure 1,** this stratagem removed most of the albumin contaminant with good recovery of fetuin. The final pellets were dissolved in 60 mM EDTA pH 7.5 and fractionated by gel filtration over Sephacryl S300HR. As shown in **Figure 2,** the major protein component recovered from this purification step has an apparent molecular weight of 59kDa, which is the same molecular weight previously found for rat fetuin. N-terminal sequence analysis of the pooled peak fractions 87-90 revealed the sequence A-P-Q-G-A-G-L-G-F-R- , which matches the N-terminal sequence of rat fetuin (19). These four peak fractions contain approximately 6mg of rat fetuin, which is 65% of the fetuin estimated to be present in the fetuin-mineral complex in the initial 16ml serum sample. As can be seen in **Table 1**, rat fetuin purified by these procedures has an average phosphorylation of 3.3 mol P/mol, which is close to the number of potential sites of serine phosphorylation by the secretory pathway protein kinase (20). In contrast, commercial samples of fetuin purified from fetal bovine serum and human serum had only 0.7 and 0.9 mol P/mol, respectively.

Biochemical characterization of the fetuin-mineral complex. A specific radioimmunoassay was developed for the quantitative detection of rat fetuin using protein

purified by the above procedures for immunoassay standards and for raising antibodies in rabbits. As shown in **Figure 3**, the fetuin radioimmunoassay detects fetuin amounts ranging from 5 to 100 ng, and the dose-dilution curve for rat serum parallels the curve for the purified rat fetuin standard.

The fetuin radioimmunoassay was used to determine the composition of the fetuin-mineral complex found in rat serum at two different etidronate doses. The complex found in serum at 6h after treatment with the 32mg etidronate/100g dose can be sedimented by centrifugation for 2h at 16,000 X g, and **Table 2** gives the amounts of calcium, phosphate, fetuin, and MGP found in the resulting pellet fraction. Assuming that the weight of the complex can be approximated by the sum of the calcium, phosphate, and fetuin weights, each 1ml of serum from rats treated with the 32mg dose of etidronate contains 1.3mg of the complex, and the complex is 46% fetuin and 53% mineral by weight.

The fetuin-mineral complex found in serum at 6h after treatment with the lower, 8mg etidronate/100g dose cannot be sedimented by centrifugation for 2h at 16,000 X g (1), and experiments were accordingly carried out to determine whether higher speed centrifugation would sediment the complex found in serum at the 8mg dose. These experiments showed that centrifugation for 1h at 164,000 X g sedimented the complex found in serum at the 8mg dose, and that the resulting pellet had the same translucent appearance as the pellet formed at the 32mg dose. **Table 2** gives the amounts of calcium, phosphate, fetuin, and MGP found in this pellet fraction. Assuming that the weight of the complex can be approximated by the sum of the calcium, phosphate, and fetuin weights, each 1 ml of serum from rats treated with the 8mg dose of etidronate contains 0.46mg of the complex, and the complex is 62% fetuin and 37% mineral by weight.

Changes in total serum fetuin levels associated with the formation and clearance of the fetuin-mineral complex. The radioimmunoassay for rat fetuin was also used to determine the time course of formation and clearance of the fetuin-mineral complex from the serum of rats treated with 32mg etidronate/100g body weight. In agreement with previous studies (1), total serum calcium increased 4 fold over the first 9h following etidronate injection, and then fell to control levels between 9 and 24h **(Figure 4A);** changes in serum phosphate paralleled the changes in serum calcium (data not shown). As seen in **Figure 4B,** centifugation of these serum samples showed that the changes in total serum calcium are due to the appearance and subsequent clearance of the fetuin-mineral complex from serum. Total serum fetuin levels were not significantly changed over the first 6h following etidronate injection (**Figure 4A)** in spite of the fact that almost half of the circulating fetuin becomes associated with the complex by the 6h time point **(Figure 4B**); this result shows that the fetuin associated with the serum mineral complex arises from the fetuin in circulation prior to etidronate injection. **Figure 4A** also shows that total serum fetuin levels fall by about 40% in the 6 to 24h interval after etidronate injection, and **Figure 4 B** shows that this reduction in total serum fetuin is associated with the disappearance of the fetuin-mineral complex from serum in the 6 to 24h interval.

Identification of additional constituents of the fetuin-mineral complex. Experiments were carried out to assess the possible presence of additional protein components in the fetuin-mineral complex. To ensure that only those proteins that are actually associated with the fetuin-mineral complex were identified, the initial pellets formed upon centrifugation of serum from etidronate-treated rats were resuspended in buffer and re-pelleted prior to analysis **(c.f. Figure 1). Figure 5A** shows the Coommassie staining pattern obtained when SDS gels were over-loaded with the fetuin-mineral complex found in rats treated with the 32mg dose of etidronate.

In order to identify protein components, the gel electrophoresis step was repeated and the proteins were transferred to a PVDF membrane. The proteins identified by N-terminal sequencing of bands cut from this membrane are shown in **Figure 5A.** In addition to the dominant fetuin component, four additional components were consistently seen in the SDS gels carried out on pellet fractions from rats treated with the 32mg etidronate dose: matrix Gla protein, secreted phosphoprotein 24, serum amyloid P component, and prothrombin. Platelet factor 4 was also consistently present in SDS gels carried out on pellets formed by centrifugation of serum from different rats but was not seen in pellets formed by centrifugation of heparin plasma from the same animals, which is consistent with the fact that plasma levels of the protein are about 1000 times lower than serum levels (21). Hemoglobin was detected in some pellets but not others; the samples that contained hemoglobin consistently had a small red component at the bottom of the pellet, which suggests that these samples may have had some erythrocyte contamination. Addition of EDTA to serum prior to centrifugation eliminated all bands associated with the fetuin-mineral complex (data not shown).

Figure 5B shows the results obtained when SDS gels were over-loaded with the fetuin-mineral complex found in the serum of rats treated with the 8mg dose of etidronate. As seen, the pattern is markedly similar to the pattern at the 32mg etidronate dose, with comparable bands of matrix Gla protein, platelet factor 4, and secreted phosphoprotein 24. Hemoglobin was present in some but not all of the serum samples, and its presence again correlated with the presence of a small red component at the bottom of the pellet. The major difference between the composition of the pellets formed by centrifugation of serum from rats treated with the 8 and 32mg etidronate doses was the presence of serum amyloid P component at the 32mg dose but not at the 8mg dose.

Additional studies were carried out to see if the addition of purified MGP, platelet factor 4, secreted phosphoprotein 24, or serum amyloid P component to serum from etidronate-treated rats prior to centrifugation would increase the level of each in the fetuin-mineral complex pellet formed after centrifugation. As seen in **Figure 6,** the addition of purified MGP, secreted phosphoprotein 24, and platelet factor 4 to serum prior to sedimentation of the fetuin-mineral complex increased the intensity of staining in the respective protein bands (lane 2). In contrast, addition of serum amyloid P component to serum did not increase the intensity of staining in this protein band **(Figure 6).** It should be noted that the addition of exogenous human platelet factor 4 increases the intensity of only one of the two platelet factor 4 bands present in the original pellet because rat platelet factor 4 is a mixture of glycosylated (9 kDa) and non-glycosylated (7 kDa) components, while the human protein consists only of a 9kDa form (22).

The importance of the vitamin K-dependent γ-carboxylation of matrix Gla protein was assessed by subcutaneous injection of 15.4mg warfarin/100g body weight 2h prior to injection of the 32mg etidronate dose. Serum was obtained 6h after etidronate injection and samples were analyzed as described for the 32mg etidronate dose in Figure 5. The resulting Coomassie-stained gel showed that warfarin treatment caused the complete loss of the MGP band, but had no effect on the intensity of the fetuin, secreted phosphoprotein 24, platelet factor 4, or serum amyloid P component bands (data not shown). These results show that the vitamin K-dependent γ-carboxylation of MGP is required for binding to the fetuin-mineral complex, and also show that the accumulation of other minor protein components in the complex is not dependent on the presence of MGP.

DISCUSSION

Composition of the fetuin-mineral complex. To our knowledge, the present studies are the first to describe the isolation of fetuin from rat serum. The procedure is rapid and simple, yielding 6mg of the purified protein from the serum of 6 etidronate-treated rats in 3 days. Since the biologically relevant serum fetuin-mineral complex is the starting point for this purification procedure and no denaturants are employed during purification, it is likely that the resulting purified rat fetuin is the biologically active, native structure of the protein. In contrast, previous studies have purified fetuin from the extracellular matrix of rat bone and have used denaturants in the purification procedures (5,9). In the present study we have used the purified, native structure of rat fetuin to make the first quantitative immunoassay for the determination of rat fetuin levels in biological fluids and extracts.

Fetuin purified from the fetuin mineral complex in rat serum by these procedures has 3 to 3.6 molecules of protein-bound phosphate. Since fetuin has not been purified from rat serum in previous studies, there is no published data on the basal phosphorylation of the total pool of serum fetuin in the rat. Serum fetuin arises from the hepatocyte, however, and previous studies have used metabolic labeling with ^{32}P to demonstrate that the fetuin secreted from rat hepatocytes is indeed phosphorylated (19,23). Although the extent of phosphorylation could not be determined by the procedures used in these earlier studies, it was shown that the phosphorylation was restricted to phosphoserine residues in the protein (23). A phosphorylated fetuin has recently been isolated from human serum and shown to contain approximately 1 mol P/mol (24). The protein-bound phosphate in this human fetuin preparation was localized to two partially phosphorylated serine residues, serine-120 and serine-312. Both serine residues lie in the sequence Ser-X-Glu/Ser(P), which is the recognition motif for phosphorylation by the

secretory pathway protein kinase (20) and is the location of phosphoserine in virtually all other presently known secreted phosphoproteins (25). These two serines are conserved in the rat fetuin, and probably account for 2 of the 3 to 3.6 moles of protein-bound phosphate found in the protein. There are two additional sites of potential serine phosphorylation by the secretory pathway protein kinase in rat fetuin, serine-310 and serine-307, and it is likely that the remaining 1 to 1.6 moles of protein-bound phosphate found in rat fetuin can be accounted for by phosphorylation of these residues.

Previous studies showed that the fetuin-mineral complex formed at the 32mg/100g weight dose of etidronate can be sedimentated by centrifugation at 16,000 X g while the complex formed at the 8mg dose cannot. We have here found that centrifugation at the higher, 164,000 X g conditions of an airfuge will sediment the fetuin-mineral complex from serum obtained from rats treated with the 8mg etidronate dose, and have used the quantitative immunoassay for rat fetuin to compare the composition of this complex to that formed at the 32mg dose. These measurements (Table 2) showed that the weight ratio of fetuin to mineral is about twice as great for complex formed at the 8mg dose than for the complex formed at the 32mg dose. The higher weight fraction of fetuin in the complex formed at the 8mg dose could account for the fact that a higher centrifugational force is needed for its sedimentation, since the density of protein is far lower than that of mineral. While the reason for the difference in fetuin to mineral ratio of the complexes formed at the 8 and 32mg etidronate doses is not presently known, one possibility is that the complex formed at the 8mg dose is smaller and so has a higher surface to volume ratio and a greater number of surface sites for fetuin binding for a given weight of mineral.

Impact of the generation and clearance of the fetuin-mineral complex on total serum fetuin levels. The present studies show that total serum fetuin levels are not significantly

changed over the first 6h following etidronate injection in spite of the fact that almost half of the circulating fetuin becomes associated with the complex by the 6h time point **(Figure 4).** The simplest explanation for this observation is that the fetuin component of the complex arises from serum fetuin. Our working hypothesis (26) is that the fetuin-mineral complex arises in the bone remodeling compartment when the acute inhibition of bone mineralization by etidronate coupled with the on-going bone resorption by osteoclasts causes a sharp rise in the concentrations of calcium and phosphate in the aqueous phase of the compartment. This rise leads to the spontaneous formation of calcium phosphate crystal nuclei whose growth is then arrested by formation of a complex with fetuin. This hypothesis is supported by the observations that fetuin inhibits the precipitation of calcium phosphate mineral phases from super saturated solutions (3), and that the fetuin-mineral complex is formed in this process (submitted). Fetuin is, however, a serum protein made in the liver and not in bone, and the question might therefore arise as to how serum fetuin can be engaged in the formation of a complex with crystal nuclei in a bone compartment. The answer to this paradox may lie in the fact that the bone remodeling compartment is itself thought to be a vascular compartment (27,28), with direct connections to the vascular system and with evidence of blood cells within the compartment. The continued flow of blood through the bone remodeling compartment could therefore supply the reservoir of serum fetuin for the continued formation of the fetuin-mineral complex within the compartment.

Previous studies showed that the fetuin-mineral complex is largely removed from blood during the 6 to 24h interval following etidronate injection (1). The present studies confirm these observations, and show for the first time that the removal of the complex from blood is associated with a reduction in total fetuin levels. Since the magnitude of this reduction is approximately the same as the amount of fetuin found in the complex at 6h **(Figure 4)**, it seems

likely that the reduction in serum fetuin during this interval is due to the removal of the fetuin-mineral complex in the 6 to 24h period. These observations indicate the existence of a specific mechanism for the removal of the entire fetuin-mineral complex from serum. While it seems likely that removal of a complex the size of the fetuin-mineral complex from blood involves receptor-mediated endocytosis, it is not clear what targets the complex for removal from blood, what receptor is involved, or which cells are engaged in removal.

Identification of other proteins associated with the fetuin-mineral complex. SDS gel electrophoresis experiments showed that the fetuin-mineral complex has a highly reproducible, simple pattern of Coomassie-stained protein bands, with bands at 10, 13, 24, 30, 59, 70, and 84kDa. N-terminal protein sequencing revealed that the 10 kDa band is the non-glycosylated form of platelet factor 4, the 13kDa band is a mixture of matrix Gla protein and the glycosylated form of platelet factor 4, the 24kDa doublet band is secreted phosphoprotein 24, the 30kDa band is serum amyloid P component, the 59kDa band is fetuin, and the 84kDa band is prothrombin; the 70kDa band could not be identified. Three of these proteins, MGP, spp24, and platelet factor 4, were found in the fetuin-mineral complex at both etidronate doses. Each of these proteins clearly has a high affinity for the complex, since the addition of each protein to serum caused the protein to become associated with the fetuin-mineral complex (Figure 6). One protein, serum amyloid P component, may not be a true component of the complex, since it was present in the complex formed at the 32mg etidronate dose but not at the 8mg dose, and since exogenous serum amyloid P component failed to accumulate on the fetuin-mineral complex.

MGP, spp24, and platelet factor 4 are minor components of serum, and the amount of each in the fetuin-mineral complex is probably evidence of their affinity for it. Previous studies have shown that serum MGP levels in untreated control rats are about 0.5

μg/ml, and rise to 20μg/ml within 9h of etidronate injection (1). Essentially all of the serum MGP in etidronate-treated rats is associated with the fetuin-mineral complex. SDS gel electrophoresis indicates that the level of platelet factor 4 and spp24 in the fetuin-mineral complex are comparable to the level of MGP, which shows that the amount of platelet factor 4, MGP, and spp24 associated with the complex in the serum of etidronate-treated rats are all probably about 20μg/ml. Since the level of platelet factor 4 in serum is also about 20μg/ml, most of the platelet factor 4 in serum is probably associated with the fetuin-mineral complex. Although the concentration of spp24 in rat serum is not known, the level in calf serum is about 1μg/ml (personal observations). The presence of far higher levels of spp24 associated with the fetuin-mineral complex than found in calf serum indicates either that rat serum contains much higher levels of the protein than calf, or that serum levels of spp24 are, like serum levels of MGP, also elevated by etidronate injection. The presence of prothrombin in the fetuin-mineral complex may be less significant, since prothrombin is a major constituent of plasma and the amount of prothrombin found in the fetuin-mineral complex consequently accounts for less than 1% of total serum levels of the protein.

Platelet factor 4 is a heparin-binding protein that is expressed by megakaryocytes and platelets and is released into serum during platelet activation. Although platelet factor 4 has been reported to have a variety of activities *in vitro*, including activity as a chemokine and as a neutrophil adhesion molecule (21), its physiological function remains obscure. It is therefore possible that platelet factor 4 could play an active role in suppressing calcification at sites of platelet aggregation where platelet factor 4 is released. However, since we could detect no platelet factor 4 associated with the fetuin-mineral complex in plasma, platelet factor 4 is

unlikely to be a significant factor in the metabolism of the fetuin-mineral complex in the general circulation.

Secreted phosphoprotein-24 has several intriguing similarities to fetuin that suggest that the proteins could have similar functions. Both proteins are synthesized in the liver and accumulate in the extracellular matrix of bone (17). Both proteins have cystatin domains, one for spp24 and two for fetuin. Both proteins have a peptide segment containing several phosphoserine residues that follows the last cystatin domain, and in both proteins the serine residues that are phosphorylated match the recognition motif for the secretory pathway protein kinase (20). Finally, both proteins have an extended C-terminal sequence following the last cystatin domain and the region of serine phosphorylation, a 62 residue segment for spp24 and a 27 residue segment for fetuin. These structural similarities between fetuin and spp24 suggest that the two proteins may have complementary functions in arresting calcification in serum.

It is intriguing to note that all three proteins that are consistent constituents of the fetuin-mineral complex, fetuin, matrix Gla protein, and secreted phosphoprotein 24, contain phosphoserine residues that match the recognition motif for the secretory pathway protein kinase (17,24,25). As isolated from bone, MGP and spp24 are partially phosphorylated at each target serine residue, however, and fetuin is not phosphorylated. We have previously speculated that incomplete serine phosphorylation in these proteins could be evidence that changes in serine phosphorylation regulate their activity in the extracellular milieu of bone (25). Because the fetuin associated with the fetuin-mineral complex is essentially fully phosphorylated, it is tempting to speculate that the fully phosphorylated form of the protein may be the active form, and that regulated dephosphorylation may occur in bone in order to inactivate its calcification inhibitory activity of the protein. This hypothesis suggests that matrix Gla protein and secreted

phosphoprotein 24 associated with the fetuin-mineral complex should also be fully phosphorylated at each target serine residue. Studies are in progress to isolate sufficient amounts of matrix Gla protein and spp24 from the fetuin-mineral complex to determine the extent of serine phosphorylation in each protein.

FOOTNOTES

1. The abbreviations used are: FMC, fetuin-mineral complex; MGP, matrix Gla protein; fetuin, α2-HS Glycoprotein; Gla, γ-Carboxyglutamic Acid; spp24, secreted phosphoprotein 24; and PF4, platelet factor 4.

REFERENCES

1. Price, P. A., Thomas, G. T., Pardini, A. W., Figueira, W. F., Caputo, J., and Williamson, M. K. (2002) *J. Biol. Chem.* 277(6), 3926-3934
2. Kazi, J. A., Nakamura, O., Ohnishi, T., Arakaki, N., Kajihara, T., Nakagawa, S., and Daikuhara, Y. (1998) *J. Biochem.* 124, 179-186
3. Schinke, T., Amendt, C., Trindl, A., Poschke, O., Muller-Esterl, W., and Jahnen-Dechent, W. (1996) *J. Biol. Chem.* 271, 20789-20796
4. Jahnen-Dechent, W., Schinke, T., Trindl, A., Muller-Esterl, W., Sablitzky, F., Kaiser, S., and Blessing, M. (1997) *J. Biol. Chem.* 272, 31496-31503
5. Mizuno, M., Farach-Carson, M. C., Pinero, G. J., Fujisawa, R., Brunn, J. C., Seyer, J. M., Bousfield, G. R., Mark, M. P., and Butler, W. T. (1991) *Bone and Mineral* 13, 1-21
6. Wendel, M., Heinegard, D., and Franzen, A. (1993) *Matrix* 13, 331-339
7. Ashton, B. A., Hohling, H. J., and Triffitt, J. T. (1976) *Calcif. Tiss. Res.* 22, 27-33
8. Ashton, B. A., Triffitt, J. T., and Herring, G. M. (1974) *Eur. J. Biochem.* 45, 525-533
9. Ohnishi, T., Arakaki, N., Nakamura, O., Hirono, S., and Daikuhara, Y. (1991) *J. Biol. Chem.* 266(22), 14636-14645
10. Quelch, K. J., Cole, W. G., and Melick, R. A. (1984) *Calcif. Tissue Int.* 36, 545-549
11. Price, P. A., and Williamson, M. K. (1985) *J. Biol. Chem.* 260, 14971-14975
12. Otawara, Y., and Price, P. A. (1986) *J. Biol. Chem.* 261, 10828-10832
13. Luo, G., Ducy, P., McKee, M. D., Pinero, G. J., Loyer, E., Behringer, R. R., and Karsenty, G. (1997) *Nature* 386, 78-81

14. Price, P. A., Faus, S. A., and Williamson, M. K. (1998) *Arterioscler. Thromb. Vasc. Biol.* 18, 1400-1407

15. Munroe, P. B., Olgunturk, R. O., Fryns, J. P., Maldergem, L. V., Ziereisen, F., Yuksel, B., Gardiner, R. M., and Chung, E. (1999) *Nature Genetics* 21, 142-144

16. Hale, J. E., Fraser, J. D., and Price, P. A. (1988) *J. Biol. Chem.* 263, 5820-5824

17. Hu, B., Coulson, L., Moyer, B., and Price, P. A. (1995) *J. Biol. Chem.* 270, 431-436

18. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) *Anal. Chem.* 28(11), 1756-1758

19. Ohnishi, T., Nakamura, O., Ozawa, M., Arakaki, N., Muramatsu, T., and Daikuhara, Y. (1993) *J. Bone and Mineral Res.* 8, 367-377

20. Meggio, F., Boulton, A. P., Marchiori, F., Forin, G., Lennon, D. P. W., Calderan, A., and Pinna, L. A. (1988) *Eur. J. Biochem.* 177, 281-284

21. Brandt, E., Petersen, F., Ludwig, A., Ehlert, J. E., Bock, L., and Flad, H.-D. (2000) *J. Leukocyte Biol.* 67, 471-478

22. Ravanat, C., Gachet, C., Hergert, J.-M., Schuhler, S., Guillemot, J. C., Uzabiaga, F., Pichard, C., Ferrara, P., Freund, M., and Cazenave, J.-P. (1994) *Euro. J. Biochem.* 223, 203-210

23. Le Cam, A., Magnaldo, I., Le Cam, G., and Auberger, P. (1985) *J. Biol. Chem.* 260, 15965-15971

24. Haglund, A. C., Ek, B., and Ek, P. (2001) *Biochem. J.* 357, 437-445

25. Price, P. A., Rice, J. S., and Williamson, M. K. (1994) *Protein Sci.* 3, 822-830

26. Price, P. A., Caputo, J. M., and Williamson, M. K. (2002) *J. Bone Mineral Res.* 17(7), 1171-1179

27. Hauge, E. M., Qvesel, D., Eriksen, E. F., Mosekilde, L., and Melsen, F. (2001) *J. Bone Mineral Res.* 16, 1575-1582

28. Parfitt, A. M. (2001) *J. Bone Miner. Res.* 16(9), 1583-1585

FIGURE LEGENDS

Figure 1. Isolation of the fetuin-mineral complex by the centrifugation of serum from etidronate-treated rats. Serum was obtained from forty-day-old male rats 6 h after subcutaneous injection with 32mg etidronate /100g body weight, and the fetuin-mineral complex was sedimented by centrifugation of this serum for 2h at 16,000 X g. After removal of the supernatants, some pellets were resuspended in HEPES buffer and centrifuged an additional 30min. Pellets from the first and second centrifugation steps were dissolved in SDS gel loading buffer containing 60mM EDTA and electrophoresed using 4 to 12% polyacrylamide gels. Lane 1, Bio-Rad low molecular weight markers. Lane 2, pellet from the first centrifugation step. Lane 3, pellet from the second centrifugation step. Lane 4, supernatant from the second centrifugation step. (see "Experimental Procedures").

Figure 2. Purification of rat fetuin by Sephacryl S-300 HR filtration of the EDTA-treated fetuin-mineral complex. Sixteen ml of serum was obtained from rats 6h after injection with 32mg etidronate/100g body weight. The fetuin-mineral complex was sedimented by centrifuging 1 ml aliquots of this serum for 2h at 16,000 X g, and the resulting pellets were resuspended in HEPES buffer and re-centrifuged. The final pellets were dissolved in 60 mM EDTA pH 7.5 and purified over a 2 X 140cm column of Sephacryl S300HR equilibrated with 5 mM NH_4HC0_3 at room temperature. Fraction size, 3ml. Inset: SDS gel electrophoresis of the fractions 87-90 on a 4 to 12% polyacrylamide gel; load, 10µg/lane.

Figure 3. Radioimmunoassay of rat fetuin and its detection of fetuin antigen in normal rat serum. Relative fraction of ^{125}I-labeled rat fetuin bound to antibody (B/Bo) at increasing amounts of purified rat fetuin ● and at increasing volumes of rat serum ○.

Figure 4. Effect of a 32mg /100g etidronate dose on serum levels of fetuin and calcium in the rat. Forty-day-old male Sprague Dawley rats were given subcutaneous injections of etidronate at a dose of 32mg/100g body weight at t=0. Panel A: blood was removed from 4 animals at the indicated times and analyzed to determine the levels of calcium and fetuin (see Experimental Procedures). Each data point is the average of the individually determined levels in the 4 experimental animals. ●, total serum calcium, mM; ○, total serum fetuin, mg/ml. Panel B: Blood was removed by exsanguination from 4 untreated control rats and from 4 rats at 6 and 24h after etidronate injection. The fetuin-mineral complex was sedimented by centrifuging serum at 16,000 X g for 2h and the resulting pellets were analyzed for fetuin and calcium as described in Experimental Procedures. Cross-hatched regions of each bar denote average supernatant levels of calcium and fetuin in the 4 rats, and open regions of each bar denote average pellet levels.

Figure 5. Identification of additional proteins in the fetuin-mineral complex. To identify additional proteins in the fetuin-mineral complex, serum and heparin plasma were obtained from rats 6h after injection with 8 or 32mg etidronate/100g body weight. The fetuin-mineral complex was sedimented by centrifuging serum or plasma at 16,000 X g for 2h (32mg dose) or at 164,000 X g for 1h (8mg dose). After removal of the supernatant, pellets were washed briefly with ice cold 0.15M NaCl and resuspended in HEPES buffer and again centrifuged at the respective centrifugal forces. The final pellets were dissolved in SDS gel loading buffer containing 60mM EDTA pH 7.5, electrophoresed on 4 to 12% polyacrylamide gels, and either stained with Coomassie or transferred to a PVDF membrane for N-terminal sequence analysis. Panel A: Electrophoresis of pellets from the indicated volume of serum or plasma from rats treated with 32mg etidronate/100g. Panel B: Electrophoresis of the pellet from 350 µl of serum from rats

treated with 8mg etidronate/100g. The proteins identified by N-terminal sequence analysis are shown.

Figure 6. Incorporation of purified matrix Gla protein, platelet factor 4, and secreted phosphoprotein 24 into the fetuin-mineral complex. To confirm the association of proteins with the fetuin-mineral complex, purified MGP, platelet factor 4, spp24, and serum amyloid P component were each added to serum obtained from rats 6h after injection with 32mg etidronate/100g body weight. As a negative control, another aliquot of the test protein was placed in an aliquot of the same serum that had been adjusted to 50mM EDTA in order to dissolve the fetuin-mineral complex. Serum was then centrifuged for 2h at 16,000 X g to sediment the fetuin-mineral complex, and the resulting pellets were dissolved in SDS gel loading buffer, electrophoresed on 4 to 12% polyacrylamide gels, and stained with Coomassie. For each gel: lane 1, Bio-Rad low molecular weight markers; lane 2, pellet from serum containing the test protein; lane 3, pellet from serum alone; lane 4, pellet from serum containing the test protein and EDTA; lane 5, the purified test protein alone.

Table 1. Comparison of the amount of protein-bound phosphate found in fetuin purified from the serum of etidronate-treated rats with that found in bovine and human fetuin. Purified rat, bovine, and human fetuin were subjected to alkaline hydrolysis and the amount of phosphate released was determined using the Malachite Green method (see Experimental Procedures).

Source of Fetuin	Moles Phosphate/Mole Fetuin	Number of Potential Serine Phosphorylation Sites[(1)]
Etidronate-treated Rat Serum	3, 3.3, 3.6[(2)]	4
Fetal Calf Serum	0.7[(3)]	6
Human Plasma	0.9[(4)]	4

[(1)] Number of serine residues having the SXE/S(P) phosphorylation recognition motif.

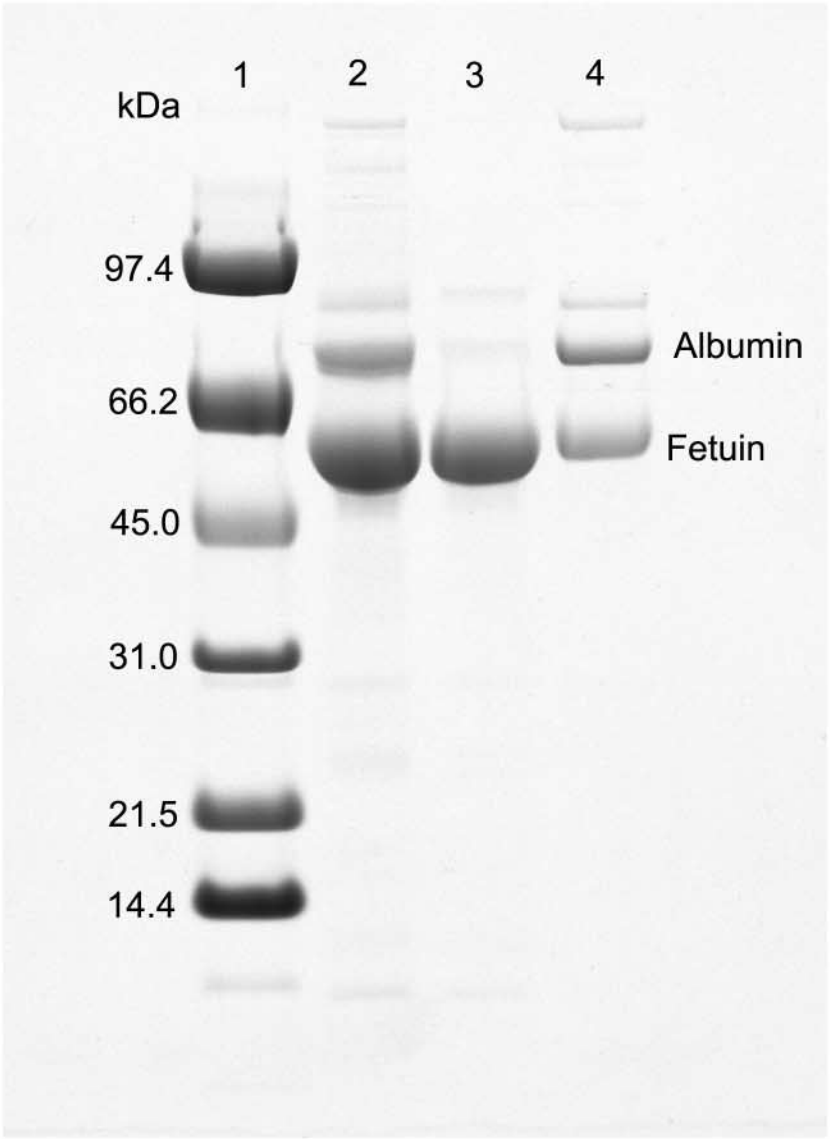
[(2)] Values obtained for 3 different preparations of fetuin purified from the serum of etidronate-treated rats.

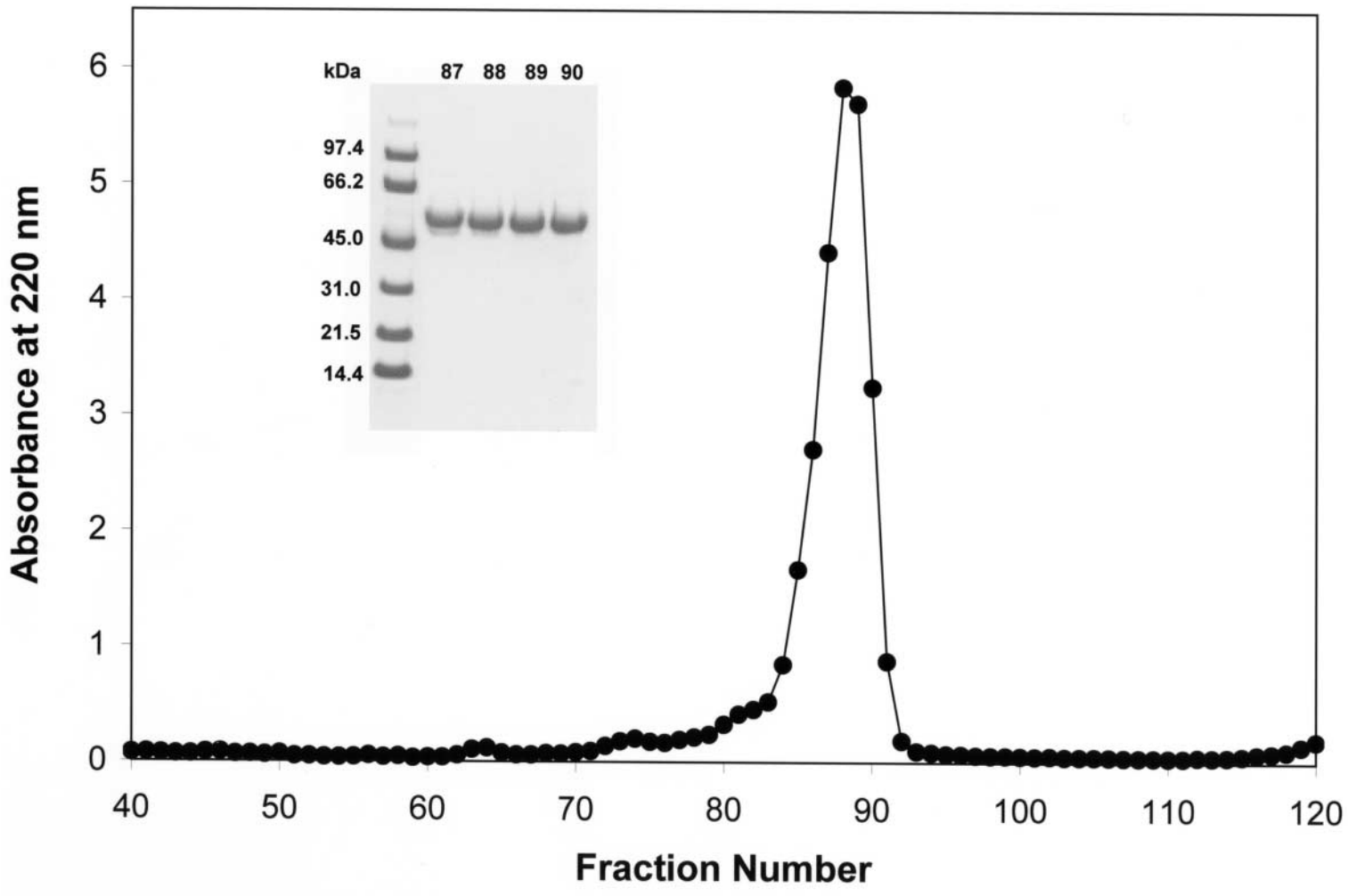
[(3)] The same value was obtained for bovine fetuin purchased from Sigma and Calbiochem.

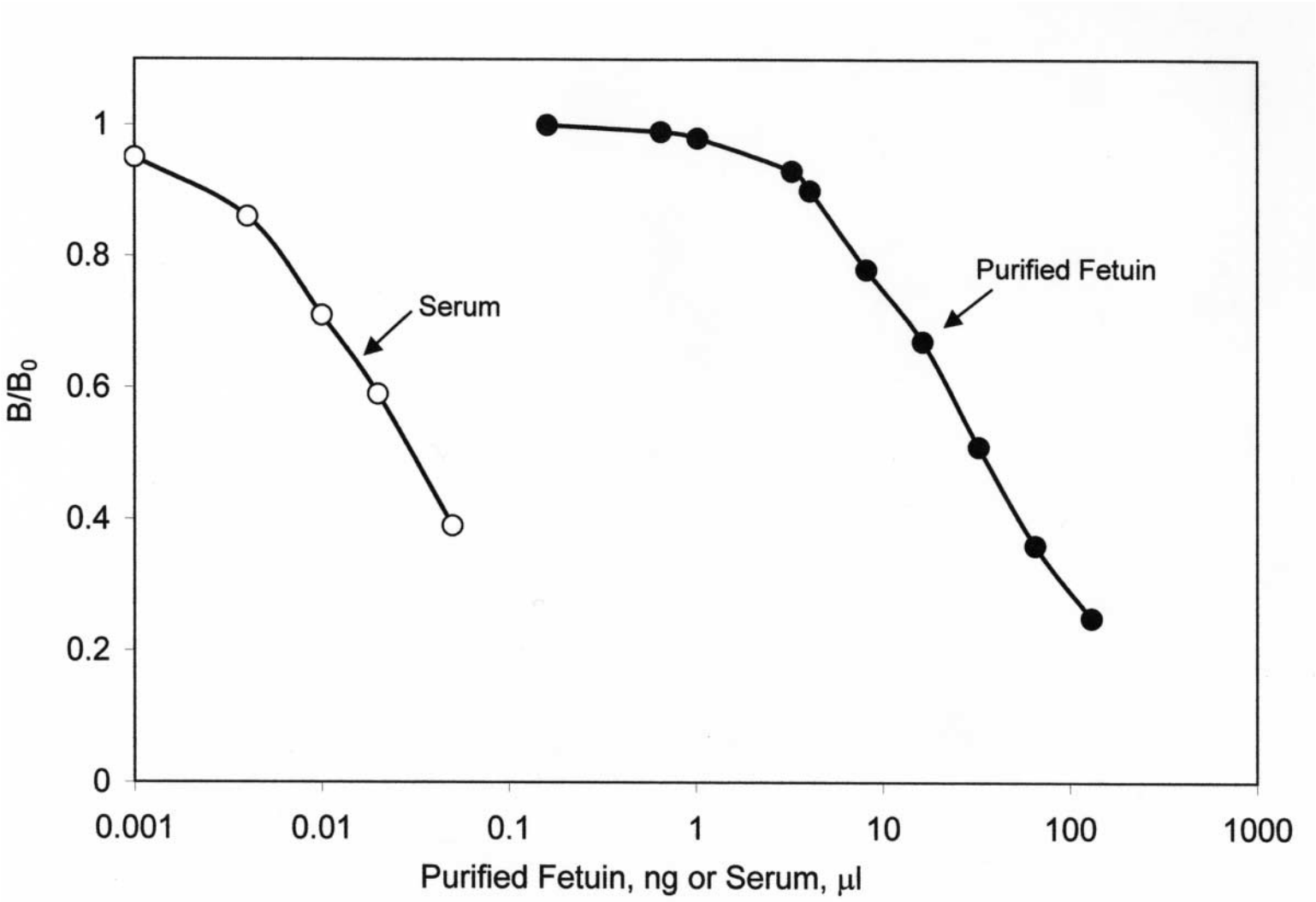
[(4)] Value obtained for human fetuin from Calbiochem.

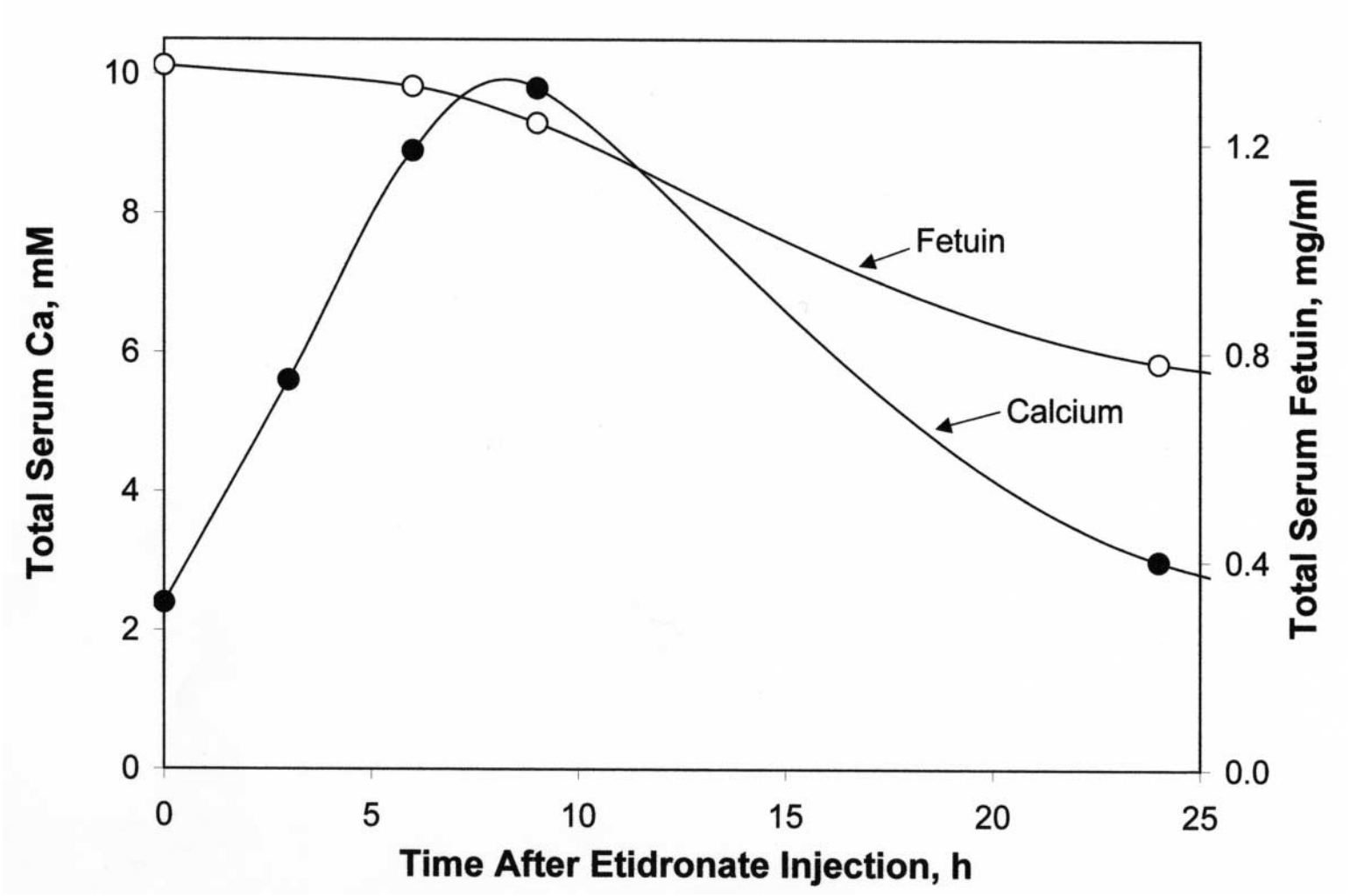
Table 2. Composition of the fetuin-mineral complex found in the serum of etidronate-treated rats. Serum was obtained from rats 6h after injection with etidronate doses of 8 mg or 32 mg/ 100g body weight. The fetuin-mineral complex was obtained by centrifugation for 2 h at 16,000 x g (32 mg dose) or by centrifugation for 1 h at 164,000 x g (8 mg dose). The resulting pellets were assayed for calcium, phosphate, fetuin and MGP. The results are expressed as the amount of complex found in 1 ml serum and are the average ± SD of three individual determinations on each serum pool (see Experimental Procedures).

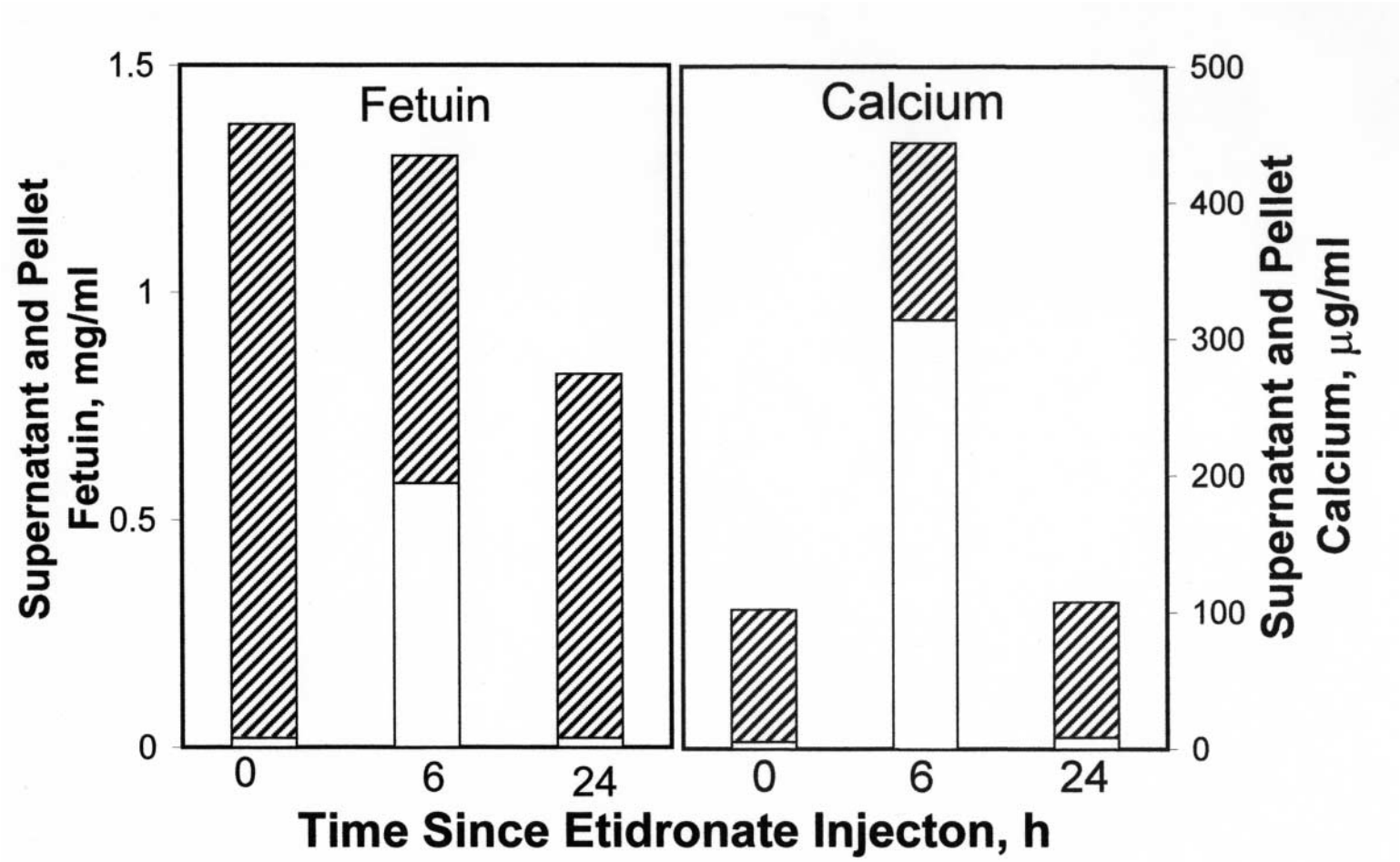
Etidronate Dose	Composition of Fetuin-Mineral Complex			
	Calcium, μg	Phosphate, μg	Fetuin, μg	MGP, μg
32 mg	314 ± 8	357 ± 41	578 ± 20	6.3 ± 1.1
8 mg	75 ± 6	97 ± 10	283 ± 30	7.2 ± 0.6

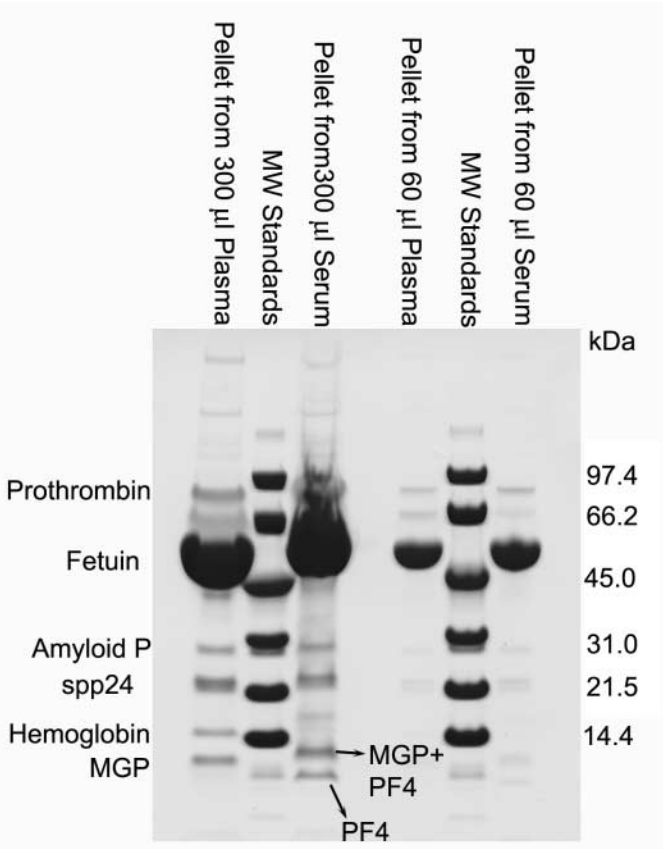

1
2
3
4
kDa
97.4
66.2
45.0
31.0
21.5
14.4
Albumin
Fetuin

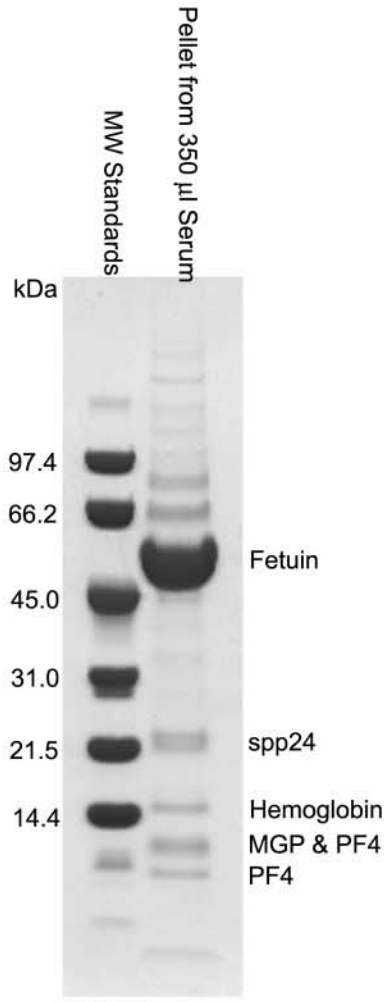

kDa
87 88 89 90
97.4
66.2
45.0
31.0
21.5
14.4
Absorbance at 220 nm
0
1
2
3
4
5
6
40
50
60
70
80
90
100
110
120
Fraction Number

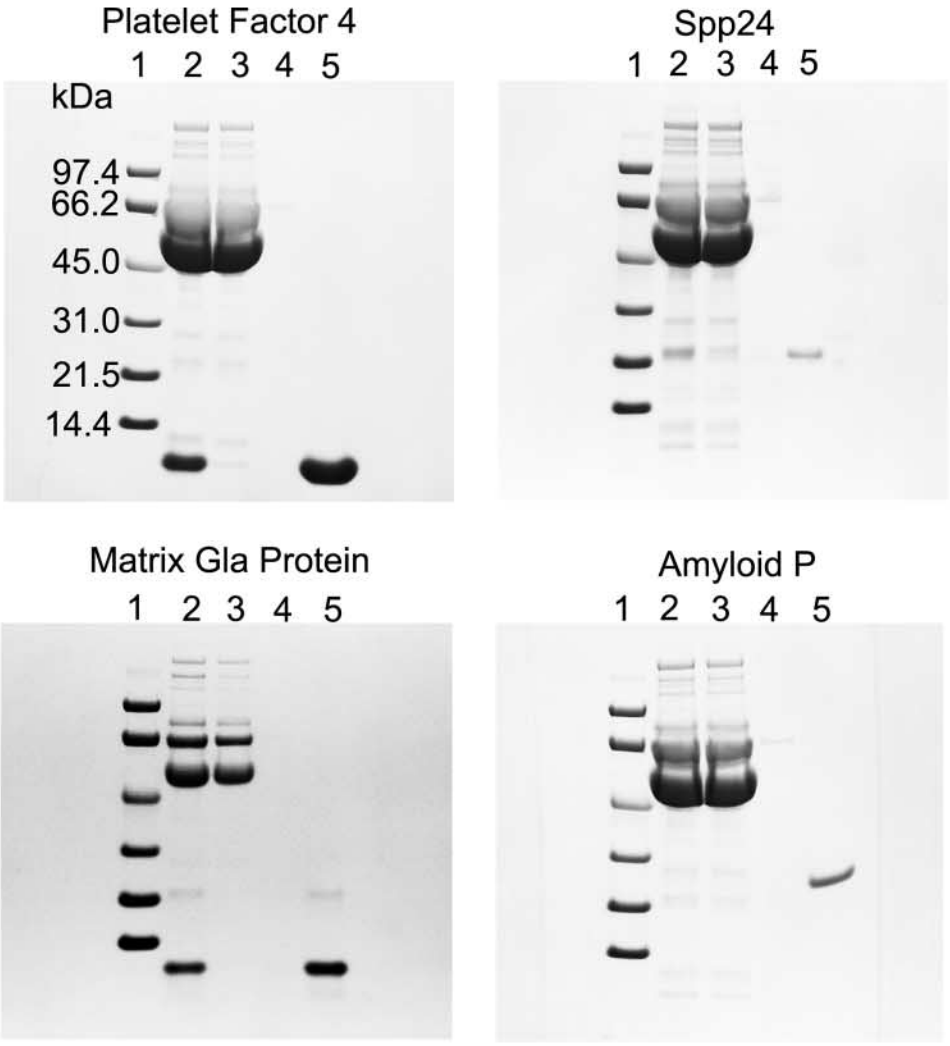

1
0.8
0.6
0.4
0.2
0
B/B0
Serum
Purified Fetuin
0.001
0.01
0.1
1
10
100
1000
Purified Fetuin, ng or Serum, µl


Total Serum Ca, mM
Total Serum Fetuin, mg/ml
Time After Etidronate Injection, h
Fetuin
Calcium
0
2
4
6
8
10
0.0
0.4
0.8
1.2
0
5
10
15
20
25


Fetuin
Calcium
Supernatant and Pellet
Fetuin, mg/ml
Supernatant and Pellet
Calcium, μg/ml
1.5
1
0.5
0
500
400
300
200
100
0
0
6
24
0
6
24
Time Since Etidronate Injecton, h


Pellet from 300 µl Plasma
MW Standards
Pellet from300 µl Serum
Pellet from 60 µl Plasma
MW Standards
Pellet from 60 µl Serum
kDa
Prothrombin
Fetuin
Amyloid P
spp24
Hemoglobin
MGP
MGP+
PF4
PF4
97.4
66.2
45.0
31.0
21.5
14.4


MW Standards
Pellet from 350 µl Serum
kDa
97.4
66.2
45.0
31.0
21.5
14.4
Fetuin
spp24
Hemoglobin
MGP & PF4
PF4


Platelet Factor 4
1 2 3 4 5
kDa
97.4
66.2
45.0
31.0
21.5
14.4
Spp24
1 2 3 4 5
Matrix Gla Protein
1 2 3 4 5
Amyloid P
1 2 3 4 5