Journal of Cellular Biochemistry 68:287–297 (1998)

ARTICLES

Inhibitors of In Vitro Mineralization From Rabbit Aorta and Their Role in Biomineralization

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Abstract Mineralization of aorta is known to occur late in life and appears to be a pathological phenomenon. In vitro studies revealed that the matrix prepared from the thoracic aorta pieces after their extraction with 3% Na_2HPO_4 and 0.1 mM $CaCl_2$ were mineralized under physiological conditions of temperature, pH, and ionic strength of the media to form matrix-bound mineral phase resembling hydroxyapatite in nature. However, the matrix identically prepared from the unextracted rabbits aortae failed to mineralize under identical assay conditions. The addition of the aorta extract in the assay system inhibited the above mineralization process. Standard biochemical techniques, e.g., dialysis, ion exchange, and molecular sieve chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and amino acid analysis by high-performance liquid chromatography were employed to isolate, purify, and characterize the potent inhibitory biomolecules from the aorta extract. The inhibitory activity of the aorta extract was found to be primarily due to the presence of three biomolecules having molecular weights of 66, 45, and 27–29 kDa. The above inhibitory biomolecules loosely associated with aorta may be involved in the control of calcification associated with arteriosclerosis. J. Cell. Biochem. 68:287–297, 1998.

Key words: aorta; mineralization; calcification; hydroxyapatite; inhibitors; arteriosclerosis

The deposition of ions in the form of a mineral phase in or on to the matrix is known as mineralization. In humans and other vertebrates in addition to bone collagen, the mineral phase has also been found to be associated with the enamel protein, aortic elastin, and matrix vesicles. The mineralization of bone and teeth is a physiological phenomenon, whereas, the mineralization of aorta is known to occur late in life and appears to be a pathological process. It is also well established that many collagenous soft tissues (e.g., tendons, skin) that do not mineralize under physiological conditions can undergo mineralization under specific pathological conditions [Cheng, 1985].

It is now widely accepted that mineralization is commonly associated with atherosclerosis and it has important clinical implications, especially in coronary arteries. Demer et al. [1994] showed that the mineral phase associated with atherosclerosis is identical to that present in bones, and several features of its development tend to indicate that it is formed not by passive precipitation, but by a mechanism similar to osteogenesis. The similarities between the mechanism of mineralization of bones and arteries, during atherosclerosis, has further been confirmed by demonstrating the presence of bone-related proteins [Roach, 1994] and osteogenic differentiation factor [Demer et al., 1994] in the artery wall.

In vertebrates, physicochemical studies have clearly shown that body fluids are supersaturated in calcium and phosphate ions with respect to hydroxyapatite, the basic mineral phase of bones, teeth (tissues that mineralize under physiological conditions), and other soft tissues known to mineralize only under specific pathological conditions [Cheng, 1985]. The question that naturally arises is why there are so few cases of pathological mineralization and why all our soft tissues do not mineralize under physiological conditions to turn us into pillars of stones. In order to explain this apparent paradox, studies have shown that body fluids contain various biomolecules which by acting either as inhibitors or promoters of mineralization may be involved in the control of mineralization of bone, teeth, and tendons [Kaiser and Bock, 1989; Weiner and Addadi, 1991; Verdier et al., 1993; Crowther and Okido, 1994; Tandon

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Received 30 May 1997; Accepted 16 September 1997

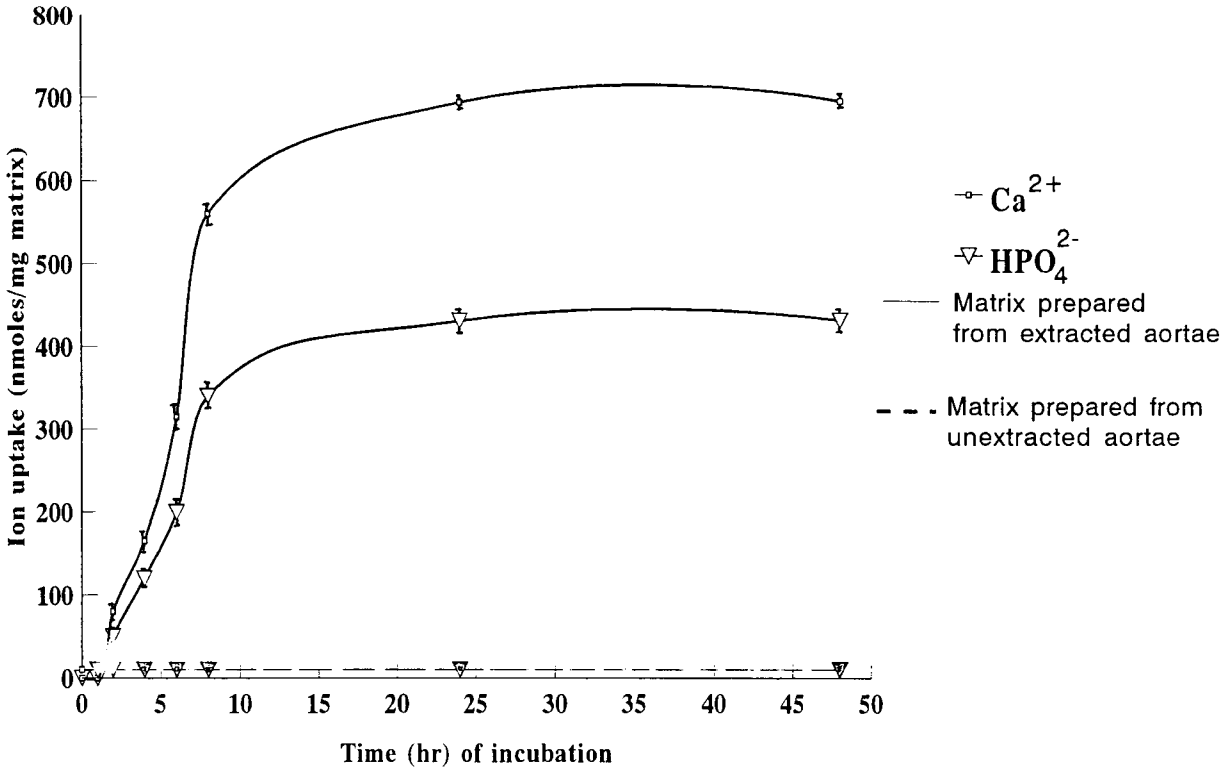

Fig. 1. Ion uptake induced by aorta matrix preparations. All values are mean ± SD of five replicates.

et al., 1997]. A review of the literature revealed that no work has been done on either the precise role of the matrix or biomolecules present in body fluids in the control of mineralization associated with atherosclerosis. Hence, the present studies were conducted to investigate the following: (1) the effect of extraction of thoracic of rabbits in 3% Na_2HPO_4 and 0.1 mM $CaCl_2$ on the ability of the matrix prepared from such thoracic aortae to mineralize under in vitro physiological conditions; and (2) the isolation, purification, and characterization of potent biomolecules from the aorta extract capable of influencing in vitro mineralization.

MATERIALS AND METHODS

Unless otherwise indicated below, all reagents and chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available.

Tissue Preparations

Rabbits procured from the Central Animal House of Panjab University were sacrificed to obtain thoracic aortae. The adventitious connective tissues were removed from the aortae with the help of a surgical blade and forcep in normal saline in 4°C. The cleaned aorta pieces were sliced into sections of approximately 1-cm-long in size and incubated at 4°C for 5–6 days in 3% $Na_2 HPO_4$ and 1×10^{-4} M $CaCl_2$ to remove loosely associated biomolecules from the aortae pieces. The method of Thomas and Tomita [1967] as modified by Jethi and Wadkins [1971] was used to prepare collagen containing fibers

TABLE I. Release of Matrix-Bound Ca^{2+} and HPO_4^{2-} Ions

Stage no.	Treatment of mineralized matrices for 1 h at 37°C	Percentage of ions released from the mineralized matrices (24 h)	
		Ca^{2+}	HPO_4^{2-}
1	Distilled water	10 ± 3	8 ± 2
2	Barbital buffer (pH 7.4)	11 ± 4	10 ± 26
3	0.1 N HCl	100	98
4	5010 mM EDTA	[a]	100

[a]Ca^{2+} could not be detected here as EDTA, employed for releasing the bound ions, interfered with the calcium determination by the method of Trinder [1960]. All values are mean ± SD of five replicates.

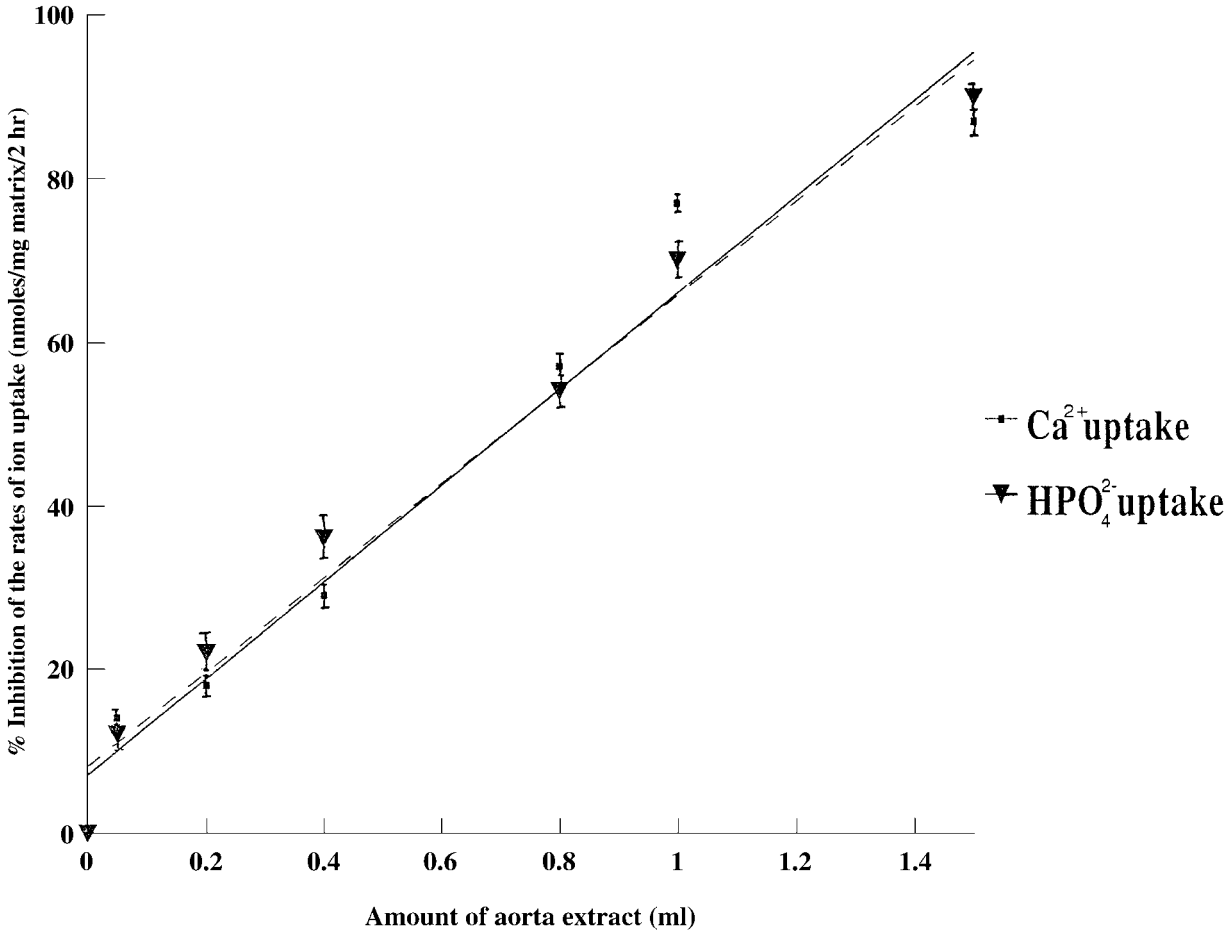

Fig. 2. Effect of thoracic aorta extract on the rates of ion uptake induced by unmineralized aorta matrix. All values are mean ± SD of five replicates.

from extracted aorta pieces. The fibers thus prepared were used as collagenous matrix for in vitro mineralization studies. The incubation medium containing soluble biomolecules removed from aortae was concentrated and used as aorta extract for isolating potent biomolecules having an ability to influence in vitro mineralization.

Heterogenous System of Mineralization

In this study, 10 mg of aorta matrix was used in the standard incubation assay system, consisting of 17.5 mM barbital buffer, 105 mM NaCl, 1.0–2.0 mM $CaCl_2$, 1.0–2.0 mM $KH_2 PO_4$ in a final volume of 5 ml [Jethi and Wadkins, 1971]. The pH of all solutions was adjusted to 7.4. The reaction was started by the addition of phosphate. Incubation was carried out at 37°C in a metabolic shaker; 0.5-ml aliquots removed at different time intervals were filtered through Whatman No. 1 papers. Net calcium and phosphate uptake from the soluble phase by the aorta matrix was determined by estimating the decrease in soluble-phase concentrations of these two ions at specific intervals of incubation. Calcium and phosphate concentrations of the samples were estimated by the methods of Trinder [1960] and Gomori [1941], respectively.

Homogeneous System of Mineralization

A mineralization system already standardized in our laboratory was used to study the extent of in vitro mineral phase formation in the absence of any matrix [Kabra et al. 1978; Singla and Jethi, 1981]. The homogeneous assay system consisted of 17.5 mM barbital buffer, pH 7.4), 105 mM NaCl, 5 mM $CaCl_2$, and 5 mM KH_2PO_4 in a final volume of 1 or 5 ml. After the addition of these reagents, the tubes were incubated at 37°C for 15 min. After the incubation the tubes were centrifuged at 4,000 rpm for 15 min to separate the precipitates. Each precipi-

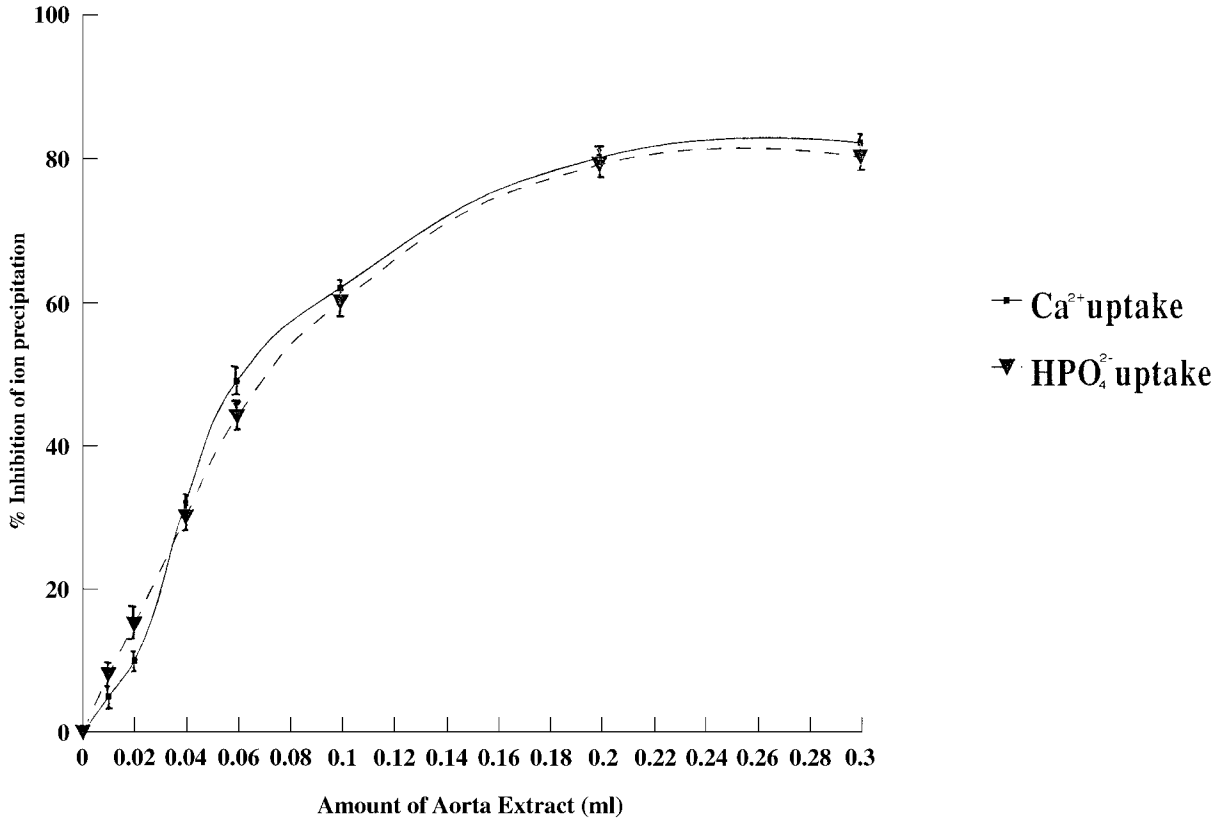

Fig. 3. Effect of aorta extract on the extent of in vitro calcium and phosphate precipitation. All values are mean ± SD of five replicates.

tate was dissolved in 0.1 N HCl to a final volume of 1 or 5 ml. The Ca^{2+} and HPO_4^{2-} concentrations in the above samples represented the extent of percipitation of these ions, which had occurred in the form of mineral phase under the given reaction conditions.

Effects of Various Test Samples on the Rates and/or Extent of In Vitro Mineralization

After adjusting the pH to 7.4, various amounts of aorta extract were added in the standard incubation systems (homogeneous or heterogeneous). Concentrations of calcium and phosphate ions in the samples were determined and proper compensations for the amounts of these ions contributed by various samples were made so as to ensure identical 0-h concentrations of these ions in all the reaction systems. Percentage inhibition or stimulation caused by various samples was calculated with respect to the control system, where identical amounts of glass-distilled water were added.

Dialysis of Aorta Extract Using Celluose Dialysis Sacs

The dialysis tubes having a cut-off molecular weight of 8 kDa were cut into 3-in.-long pieces and used for the dialysis of aorta extract [McPhie, 1971]. A known amount of extract was transefered into each of the dialysis tube and the dialysis was conducted against glass-distilled water with constant stirring at 4°C in a cold room. After every 24 h, the water was exchanged with fresh distilled water, and after 72 h, each tube was removed and various dialysates and dialysants recovered at different intervals were concentrated to a known volume using Eyela lyophilizer and their protein concentrations were determined [Lowry, 1951].

Purification and Characterization of Potent Biomolecules From Aorta Extract

DEAE-cellulose chromatography, molecular-sieve chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and amino acid analysis by high-performance

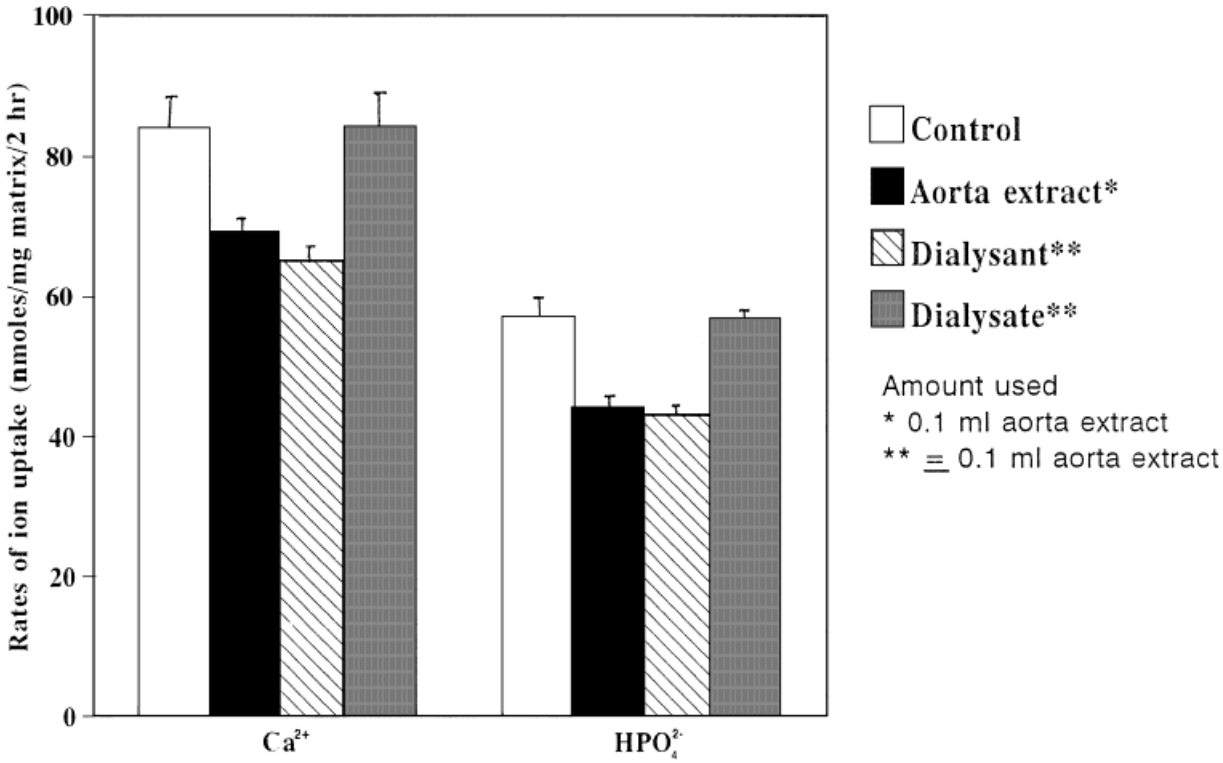

Fig. 4. Effect of dialysis on the ability of aorta extract to influence the rates of ion uptake induced by aorta matrix. All values are mean ± SD of five replicates.

liquid chromatography (HPLC) were employed to purify and characterize the potent biomolecules.

RESULTS

Effect of Extraction on the Mineralizing Ability of the Aorta Matrix and the Influence of Extraction Media on the Process of Heterogeneous Assay System

When the matrix prepared from unextracted thoracic aortae was incubated in a standard assay system, no significant uptake of either Ca^{2+} or HPO_4^{2-} was observed (Fig. 1). However, the matrix prepared after extraction of aortae with 3% Na_2HPO_4 and 0.1 mM $CaCl_2$ was found to induce the uptake of Ca^{2+} and HPO_4^{2-} ions from the stable solutions to form a matrix-bound mineral phase. After 1 h of incubation, a progressive uptake of Ca^{2+} and HPO_4^{2-} ions from the reaction system was observed, and the uptake continued until a limiting velocity was attained at about 24 h. The mM Ca^{2+} mM HPO_4^{2-} product in the reaction system attained at the limiting velocity was found to be approximately equal to 0.48 mM^2. Prolonging the incubation further even up to 48 h did not result in any additional uptake of either ion from the reaction system. The ratio at which Ca^{2+} and HPO_4^{2-} ions disappeared from the media at various times of incubation was found to within a range of 1.50–1.61:1. The ions that disappeared from the reaction system after attainment of the limiting velocity were found to be tightly associated with the matrix. Treatment of the mineralized matrix with either distilled water or barbital buffer failed to release the ions from their matrix association. However, these ions were found to be completely released from their matrix association by treating the mineralized matrices with 0.1 N HCl or EDTA only (Table I). The studies (Fig. 2) further revealed that when aorta extract (obtained during preparation of matrix from thoracic aorta) was added in the reaction system it was found to significantly inhibit the rates of both Ca^{2+} and HPO_4^{2-} uptake from the soluble phase. The addition of 0.05, 0.1, 0.4, 0.8, 1.0, or 1.5 ml of aorta extract in the reaction system were found to inhibit the initial rates of Ca^{2+} and HPO_4^{2-} uptake (nmoles/mg matrix 2 h) by 14%, 18%, 29%, 57%, 76%, or 88% and 12%, 22%, 36%, 56%, 72%, or 89% respectively.

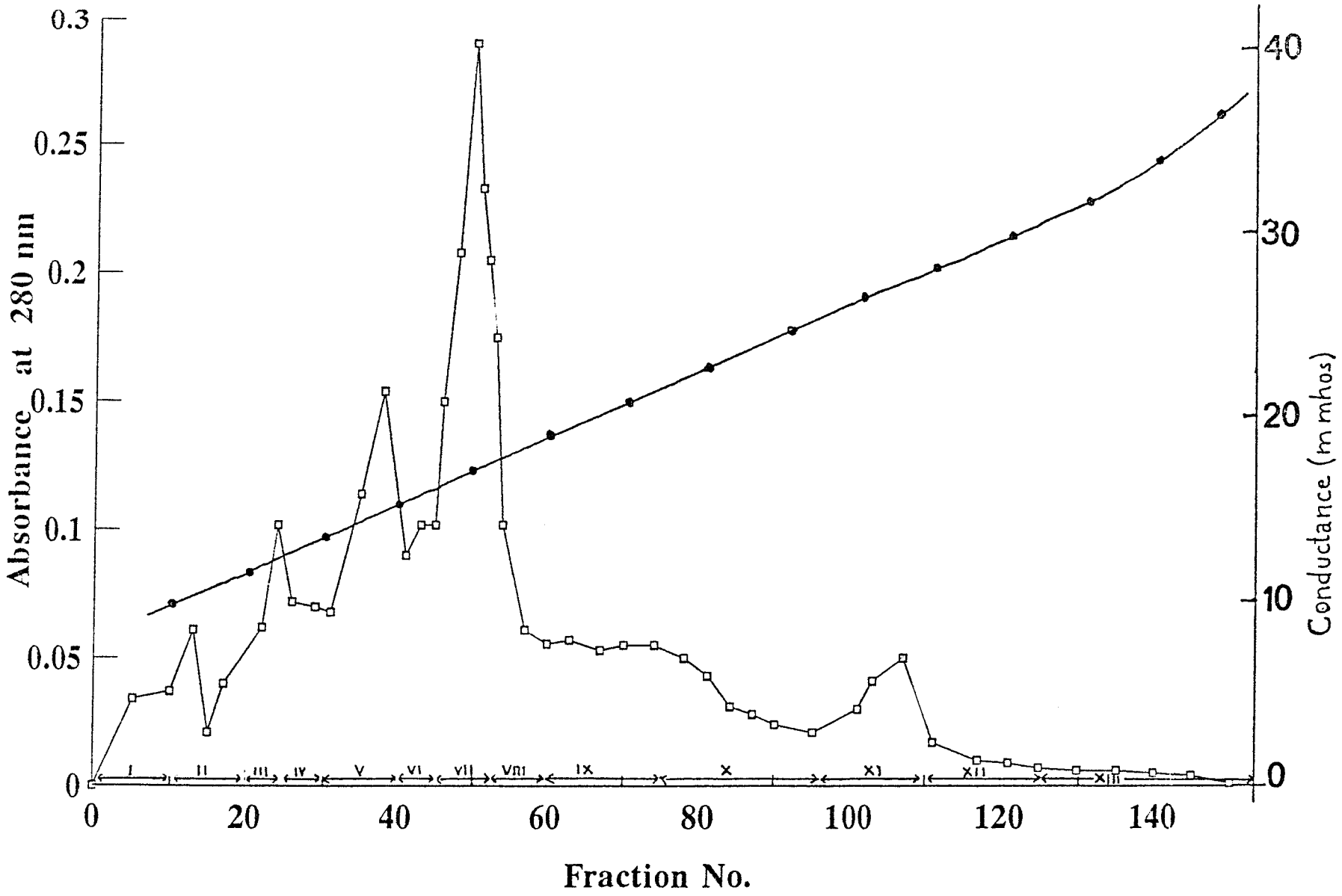

Fig. 5. Fractionation of dialysant (>8 kDa) on DEAE-cellulose column. -●-, conductance; -□-, absorbance.

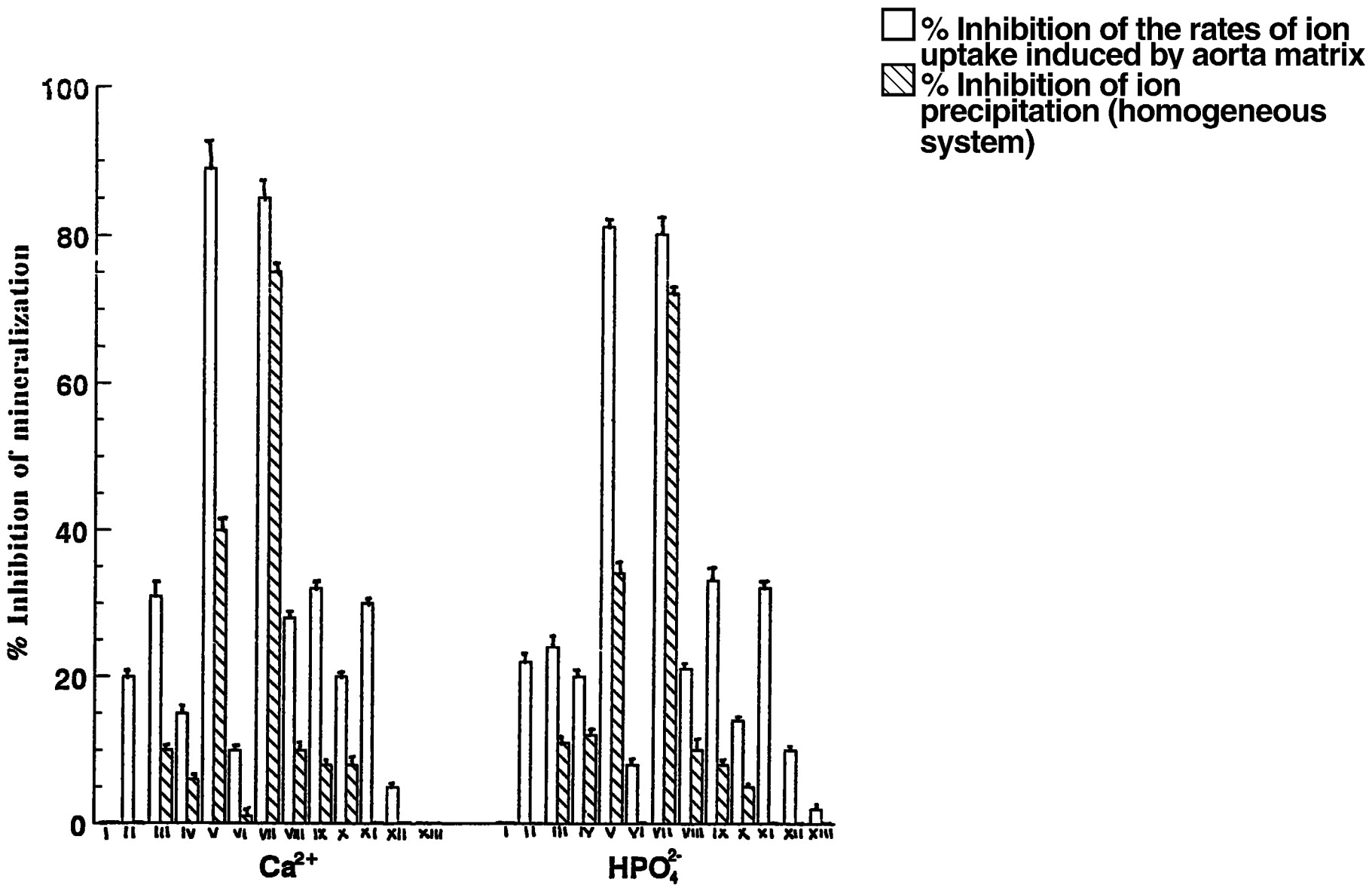

Fig. 6. Effect of grouped DEAE-cellulose fractions of the aorta extract dialysant (>8 kDa) on mineralization. All values are mean ± SD of five replicates.

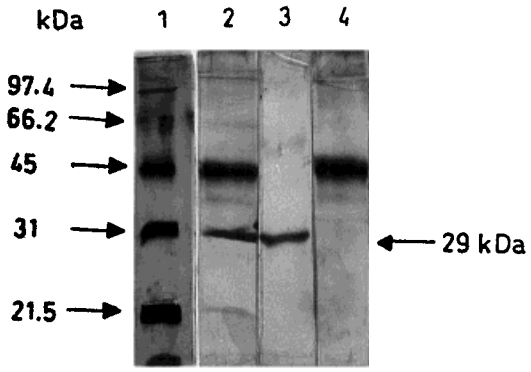

Fig. 7. SDS–PAGE of DEAE-cellulose fraction V (**lane 2**) and G-50 fractions F1 (**lane 4**) and F2 (**lane 3**), stained with silver nitrate.

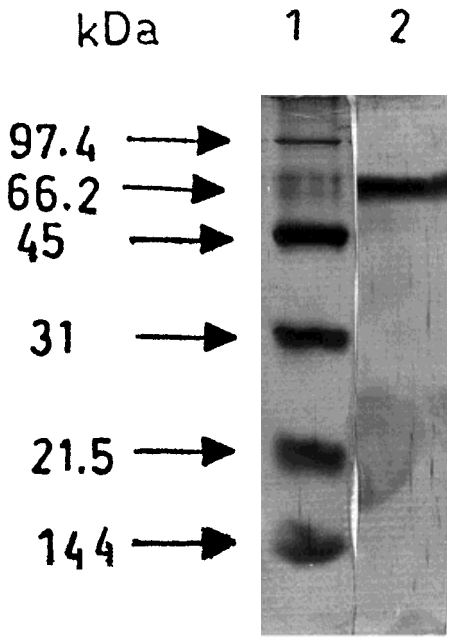

Fig. 8. SDS–PAGE of DEAE-cellulose fraction VII (**lane 2**), stained with silver nitrate.

Effect of Aorta Extract on In Vitro Mineralization in the Absence of the Matrix in the Homogeneous Assay System

The results presented in Figure 3 demonstrate that, similar to the studies with heterogeneous system, the biomolecule(s) present in the thoracic aorta extract were also able to inhibit the precipitation of Ca^{2+} and HPO_4^{2-} ions in the form of a mineral phase, in the absence of an organic matrix (homogeneous system). With the increase in the volume of the aorta extract added to the assay system, a progressive increase in the inhibitory activity was observed. The addition of 0.01, 0.02, 0.04, 0.06, 0.10, 0.20, or 0.30 ml of aorta extract in the reaction were found to inhibit the extent of Ca^{2+} and HPO_4^{2-} precipitation by 5%, 10%, 32%, 49%, 62%, 80%, or 82% and 8%, 15%, 30%, 44%, 66%, 79%, or 80%, respectively.

Purification and Characterization of Potent Inhibitory Biomolecules From Aorta Extract

Dialysis of aorta extract against distilled water using dialysis tubes having a cutoff molecular weight of 8 kDa revealed that all the inhibitory potency associated with the thoracic aorta extract could be recovered from the dialysant (>8-kDa fraction) of the aorta extract (Fig. 4).

Studies further revealed that when dialysant was subjected to anion-exchange chromatography using DEAE-cellulose column and the fraction eluted by a linear Tris–NaCl gradient (5×10^{-2} to 0.5 M), the inhibitory potency of dialysant was found to be mainly due to the presence of at least two biomolecules represented by grouped fractions V and VII (Fig. 5). Identical conclusions were drawn when either heterogeneous or homogeneous systems of in vitro mineralization were employed to assay the inhibitory potencies of various DEAE-cellulose fractions (Fig. 6).

SDS–PAGE of fractions V and VII revealed that the inhibitory potency of fraction V was due to the presence of two biomolecules having molecular weights equal to 45 and 29 kDa (Fig. 7), and the inhibitory potency of fraction VII was due to one biomolecule of molecular weight 66 kDa (Fig. 8). The heterogeneity of the DEAE-cellulose fraction V was further confirmed by its further purification on G-50 column (0.05 M Tris–NaCl buffer; pH 7.4). The results presented in Figure 9 indicated that fraction V indeed contains two biomolecules. Standardization of the column with low-molecular-weight proteins (Sigma) indicated that the molecular weights of the biomolecules represented by G-50 fractions F1 and F2 were 45 and 27 kDa. This was reconfirmed by SDS–PAGE as well (Fig. 7). Identical trends regarding the inhibitory potencies of various G-50 fractions were obtained when either a heterogeneous or homogeneous system of in vitro mineralization was employed to assay various fractions (Fig. 10).

DISCUSSION

The matrix prepared from rabbit thoracic aortae after extraction with the Na_2HPO_4–$CaCl_2$ mixture was found to induce the uptake

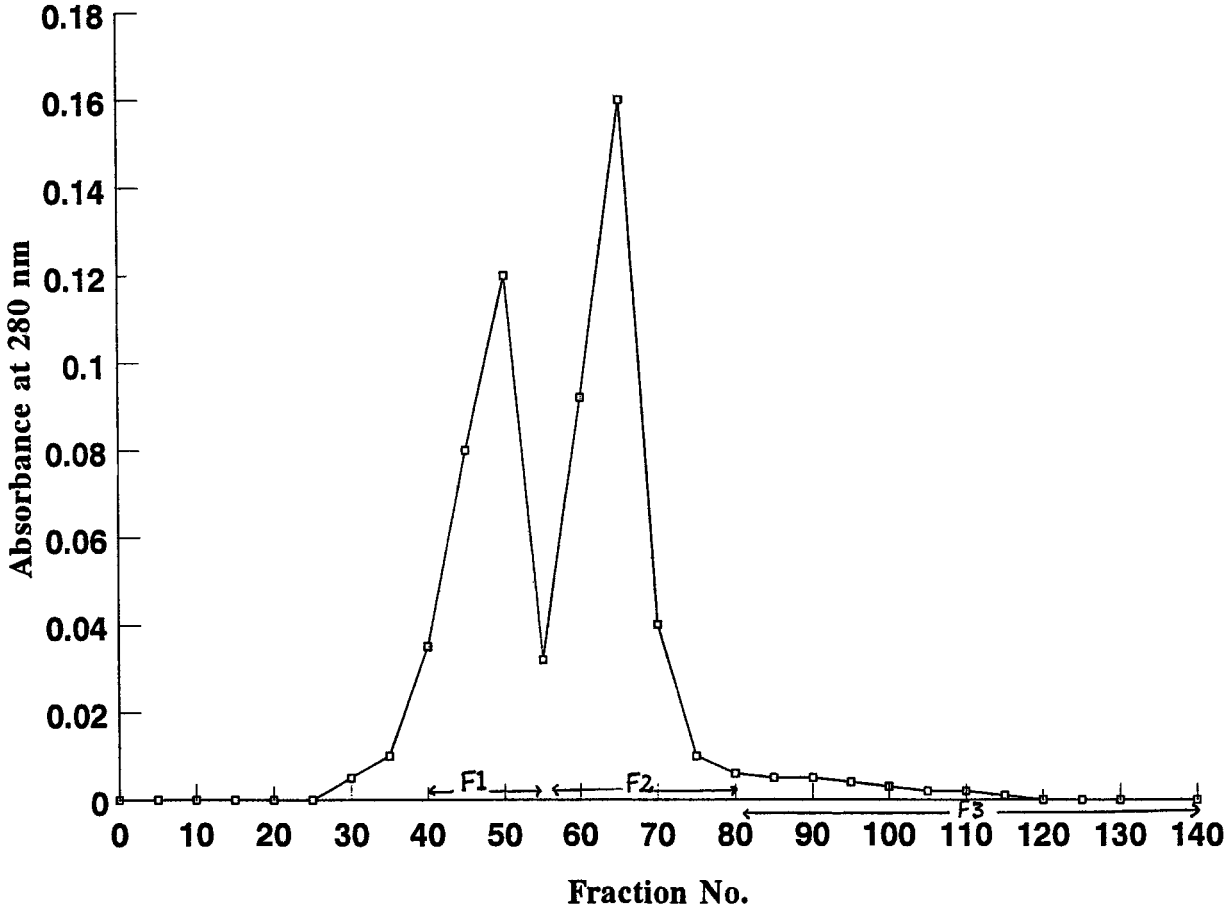

Fig. 9. Fractionation of grouped DEAE-cellulose fraction V on G-50 column.

of Ca^{2+} and HPO_4^{2-} ions from the reaction system to form a matrix-bound mineral phase. However, the matrix prepared from the unextracted aortae failed to undergo in vitro mineralization. Although no attempt was made to determine directly the chemical nature of the mineral phase formed during the present studies using physical tools, yet the ratio of the Ca^{2+} and HPO_4^{2-} present in the mineral phase and the similarity between the $Ca^{2+} \times HPO_4^{2-}$ product attained at limiting velocity with the solubility product of these ions in bone mineral phase [Levinskas and Neuman, 1955; Nordin, 1957] strongly suggest that the mineral phase resembles hydroxyapatite in nature. Aorta extract was found to contain biomolecules having the ability to act as potent inhibitors of in vitro mineralization both in the presence of an organic matrix (heterogeneous system) and in the absence of an organic matrix (homogeneous system). It would not be fair to compare directly the inhibitory potencies of various inhibitory biomolecules between heterogeneous and homogeneous systems, as it has been clearly shown in kinetic studies that the mechanisms of in vitro heterogeneous and homogeneous mineralization is entirely different [Jethi and Wadkins, 1971]. The inhibitory potency of the aorta extract was found to be due to the presence of biomolecules having molecular weights of >8 kDa. Purification and characterization of the potent inhibitory biomolecules revealed that three biomolecules having molecular weights of 66, 45, and 27–29 kDa were indeed responsible for the inhibitory activity of the aorta extract. Amino acid analysis of these inhibitory biomolecules (Table II) revealed that aromatic, basic and acidic amino acids accounted for 32.6%, 28.6%, and 14.2%, respectively, of the total amino acids of 66 kDa inhibitor. Amino acid analysis of 45 and 27–29-kDa inhibitory biomolecules revealed that both inhibitors contained a highest percentage of basic amino acids. Basic amino acids were found to constitute 29.2% and 43% of these inhibitors, respectively. Aromatic amino acids were found to constuite 18.3% and

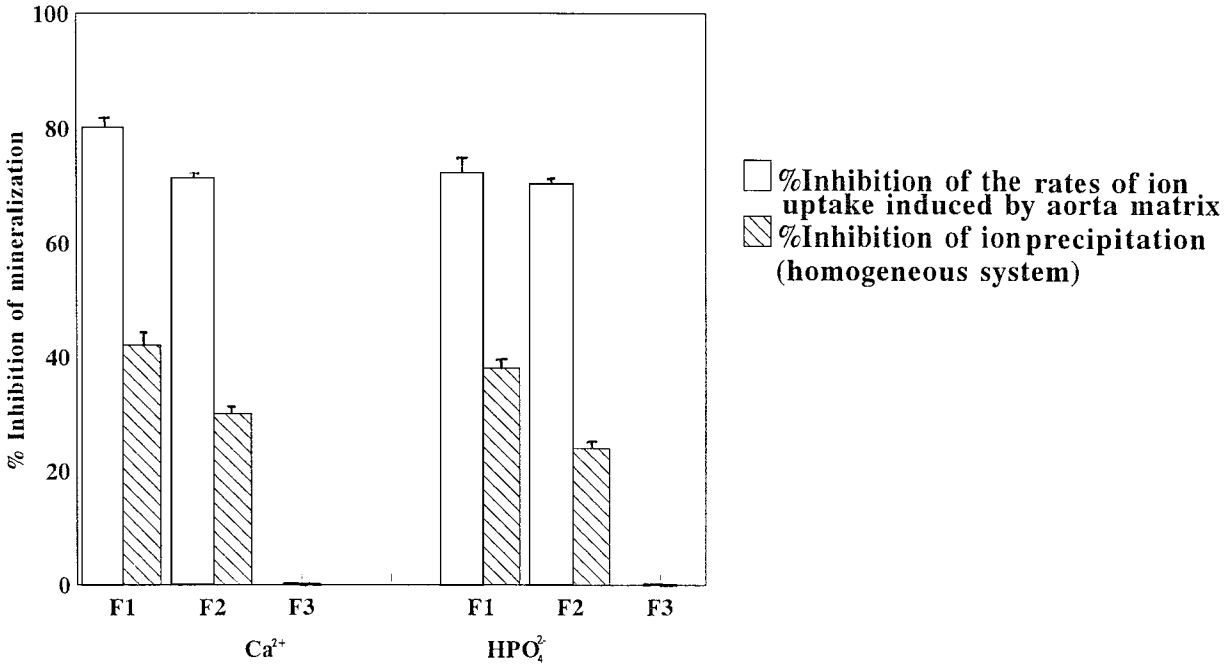

Fig. 10. Effect of G-50 grouped fractions of DEAE-cellulose grouped fraction V on mineralization. All values are mean ± SD of five replicates.

TABLE II. Amino Acid Composition of Purified Fractions

	% Composition		
	DEAE-cellulose fraction VII	G-50 fractions of DEAE-cellulose fraction V	
Amino acids	(66 kDa)	F1 (45 kDa)	F2 (27–29 kDa)
Aspartic acid	4.0	3.0	—
Glutamic acid	10.2	3.2	7.2
Serine	—	—	—
Glycine	0.8	10.0	12.2
Histidine	—	4.0	10.0
Arginine	26.0	10.0	15.0
Threonine	—	2.0	—
Alanine	0.3	3.5	5.0
Proline	7.4	14.8	10.8
Tyrosine	27.1	14.0	9.2
Valine	1.0	—	—
Methionine	9.6	4.6	—
Cysteine	0.9	—	—
Isoleucine	1.8	5.0	7.1
Leucine	2.6	6.1	4.0
Phenylalanine	5.5	4.3	1.3
Lysine	2.6	15.2	18.0

10.5% of 45 and 27–29 kDa inhibitor, respectively. Both inhibitors were found to be poor in acidic amino acids.

Similar to the studies with aorta, potent inhibitory biomolecules have been found to be loosely associated with flexor tendons of rabbits [Tandon et al., 1997]. It is interesting to note that the three inhibitory proteins isolated from the parotid saliva of rabbits were found to have molecular weights of approximately 66, 42, and 30 kDa [Spielman et al., 1990]. This apparent resemblance among the three inhibitory biomolecules isolated from thoracic aorta extract of rabbit during the present studies with the three inhibitory biomolecules from parotid saliva assumes practical evolutionary physiological significance in the control of physiological/pathological mineralization.

Keeping in view the above findings and the similarities observed between the mechanism of mineralization of bones and calcified human atherosclerotic lesions, it is highly likely that a unique unifiying mechanism involving bioregulatory inhibitory molecules may exist that can regulate biomineralization under physiological and pathological conditions.

The demonstration of loosely bound inhibitors with aorta and tendons raises a strong possibility about the role of these inhibitors in

the control of biological mineralization. The mineralization of aorta and tendons under specific pathological conditions could perhaps be attributed to the dissociation or inactivation of these potent inhibitory biomolecules.

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