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Inhibitory activity of bovine milk osteopontin and its fragments on the formation of calcium phosphate precipitates

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Osteopontin (OPN) is an acidic phosphorylated glycoprotein found in many tissues and physiological fluids. OPN is known to possess multiple biological functions including bone remodeling, cell adhesion, cellular transformation and immunological mediation; these functions have been extensively reviewed (Butler et al. 1996; Sodek et al. 2000).

In mammary gland, the involvement of OPN in mammary differentiation and involution has been proposed (Rittling & Novick, 1997; Nemir et al. 2000). The biological significance of OPN in mammary secretion remains unclear, however, Nagatomo et al. (2004) predicted a contribution of OPN in human milk to the immunological development of breast-fed infants. They observed an increase of OPN concentration as lactation proceeded, and it was not unusual to find the concentration of OPN in human milk exceeding in 1 g/l. In contrast, the abundance of OPN in bovine mammary secretion is recognized at the early stage of lactation rather than in matured milk, and the concentration of OPN in mature bovine milk is estimated to be about 10 mg/l (Kumura et al. 2004). Thus, the level of OPN is much higher in human milk than in bovine milk.

Christensen et al. (2005) reported the post-translational modification of 36 phosphorylation sites in human milk OPN while Sørensen et al. (1995) reported that mature bovine milk OPN consisted of 262 amino acids and was phosphorylated at 27 serine residues and one threonine residue. Considering its availability, bovine OPN is an attractive source for OPN. In fact, OPN can be prepared easily from milk or whey (Bayless et al. 1997; Azuma et al. 2006). When intact OPN is required, acid whey should be used because OPN is susceptible to chymosin (Kumura et al. 2004). Traditional cheese making practice leads to fragmentation of OPN due to chymosin and results in release of a variety of phosphorylated peptides in cheese whey since phospholylated amino acid residues are distributed throughout the OPN molecule.

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Among the phosphorylated peptides derived from milk, casein phosphopeptide (CPP), which could be obtained by exposure of casein to intestinal digestion, is well known and possesses a high calcium binding capacity that prevents the precipitation of calcium phosphate salts. CPP is regarded as a functional food ingredient that increases the soluble intraluminal calcium availability for absorption across the mucosa (Naito, 1986; Kitts & Yuan, 1992) and is authorized as a food for specified uses (FOSHU) by the Japanese Ministry of Health, Labour and Welfare.

In this study, we observed inhibitory activity of OPN and its fragment on the formation of calcium phosphate precipitates. Comparative study of its activity with CPP was also conducted, and the sequence in OPN molecule responsible for its activity is discussed.

Materials and Methods

Preparation of CPP, OPN and OPN digest

CPP was prepared according to the procedure of Hirayama et al. (1992). In brief, whole casein (pH 7·0) was digested with trypsin, followed by adjustment to pH 4·5. The preparation was centrifuged to obtain the supernatant. Phosphopeptides in the solution were enriched by precipitation of the peptides with the addition of $CaCl_2$ and ethanol. The precipitate was washed with 50% ethanol and air-dried to use as CPP.

Isolation of OPN from bovine milk was carried out as described previously (Kumura et al. 2004). The purified OPN (1·6 mg/ml) was dissolved in 0·01 M-glycine-HCl buffer, pH 2·5, or 0·01 M-Tris-HCl buffer, pH 7·5, to be digested with trypsin or pepsin, respectively. The reaction was carried out at 37 °C for a defined incubation period, followed by heating at 80 °C for 15 min. Dual treatment, consisting of incubation with trypsin followed by peptic digestion, was also performed; reactions were carried out at 37 °C for 3 h for each enzyme. Enzyme used in this study was purchased from Sigma Chemical Co. (St. Louis, MO 63178, USA), and all enzymatic reactions

were carried out at a weight ratio of OPN to enzyme of 50:1. A minimal amount of NaOH or HCl was added to the resulting solutions to adjust the pH value to 7·0.

Digested OPN samples were loaded onto 12·5% polyacrylamide gel containing 1 g SDS/l according to the procedure of Laemmli (1970) to confirm degree of degradation.

Proteins in the gel were stained with 1-ethyl-2-[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methylpropenyl] naphtho[1,2d]-thiazolium bromide known as ''Stains-All'' (Eastman Organic Chemicals, New York, NY 14650 USA) according to the procedure of Green et al. (1973).

Protein determination

The protein concentration of the purified OPN solution was spectrophotometrically determined. It was first estimated by a modified Lowry method (Bensadoun & Weinstein, 1976) using bovine serum albumin as a standard; the absorbance of the resulting OPN (1 mg/ml) solution at 280 nm corresponded to a value of 0·667.

HPLC

The purified OPN, treated with pepsin at 37 °C for 24 h, was loaded on a CAP CELL PAK C8 column (4·6 × 150 mm; Shiseido Co., Ltd., Ginza, Japan). The experimental conditions were as previously described (Kumura et al. 1999).

Inhibitory effect of OPN, OPN digests and CPP on the formation of Ca-phosphate-precipitates

The inhibitory activity of OPN on the formation of calcium phosphate precipitate was determined by the method of Naito (1986). In brief, a solution of 0·04 M-$CaCl_2$ was added to an equal volume of OPN solution, OPN digests, or CPP, and 0·25 ml of the resulting mixture was added to 1 ml 0·02 M-sodium phosphate buffer, pH 7·0. After vigorous mixing, the mixture was incubated at room temperature for a defined period. The supernatant was obtained by centrifugation at 10 000 × ***g*** for 1 min, and the soluble calcium concentration in the supernatant was determined using a commercial kit (Calcium-C test, Wako Pure Chemical Industries, Osaka, Japan). The experiment was performed in triplicate and repeated three times.

Further fractionation of OPN digested with pepsin was performed using HPLC. The obtained fractions were centrifuged under vacuum conditions to remove acetonitrile. Deionized water was added to each fraction to adjust the equivalent volume to the initially injected sample volume of the HPLC column. The inhibitory activity of each fraction was examined using a single tube, and the experiment was repeated four times.

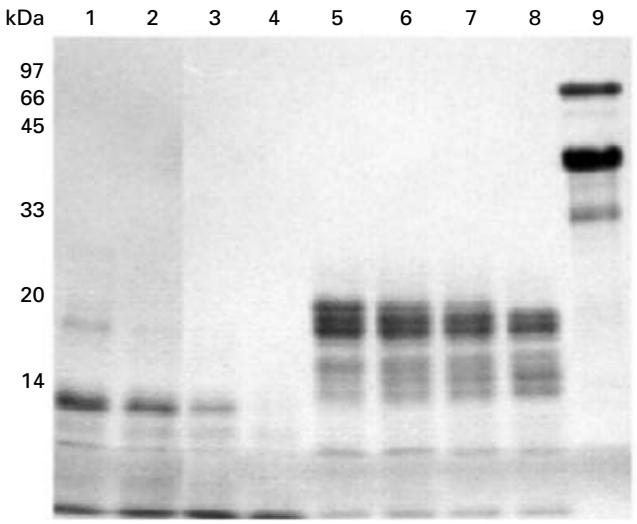

Fig. 1. Sensitivity of OPN to pepsin and trypsin. Samples were loaded on each lane for SDS-PAGE and stained with Stains-All. OPN incubated with pepsin for 1 h (lane 1), 3 h (lane 2), 6 h (lane 3), 24 h (lane 4); OPN incubated with trypsin for 1 h (lane 5), 3 h (lane 6), 6 h (lane 7), 24 h (lane 8); intact OPN (lane 9).

MALDI-TOF-MS and N-terminal amino acid sequence analysis

Mass spectrometry of peptides, obtained by HPLC, was analysed using MALDI-TOF-MS (Voyager DE STR, Applied Biosystems, Foster City, CA 94404 USA) according to the manufacturer's instructions.

The N-terminal amino acid sequence of the fractions obtained by HPLC was analysed using a protein sequencer (model 492A, Applied Biosystems).

Peptide synthesis

The synthetic peptides used in this study were purchased from Thermo Electron (Ulm, Germany).

Results

When OPN was digested with pepsin or trypsin for 1 h, the molecular mass was reduced to less than 14 kDa or 22 kDa, respectively (Fig. 1). Further treatment with pepsin continued the gradual increase of OPN degradation; however, further treatment with trypsin was ineffective. Thus, OPN proved more resistant to trypsin than to pepsin.

The inhibitory effect of intact OPN and CPP on the formation of calcium phosphate precipitates is shown in Fig. 2. A negligible amount of endogenous calcium was estimated in both CPP and purified OPN preparations. The effect of CPP was maintained for 1 h when its concentration was 160 μg/ml; however, no effect was detected when a lower concentration of CPP (32 μg/ml) was used. In contrast, the inhibitory effect of OPN at 160 μg/ml was sustainable for 3 h. Even with a lower concentration

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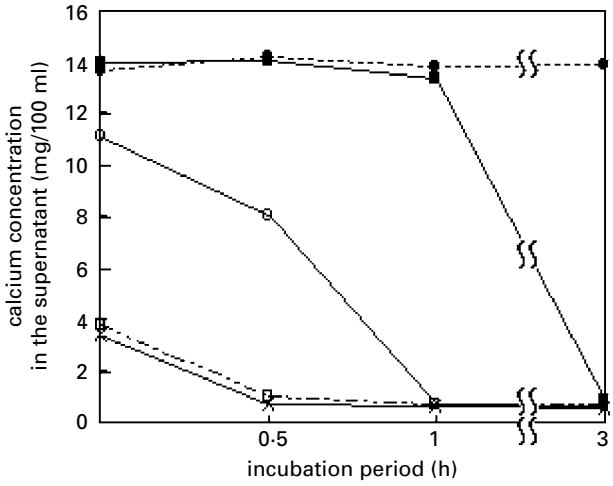

Fig. 2. Inhibitory activity of OPN and CPP on the formation of calcium phosphate precipitates. Calcium chloride solution in the absence or presence of CPP or OPN was mixed with sodium phosphate solution and incubated for a defined period at room temperature. Calcium concentration in the soluble fraction was determined. Final concentration was 32 μg/ml (CPP; □, OPN; ○), 160 μg/ml (CPP; ■, OPN; ●). A control (×) that contained neither CPP nor OPN was also used.

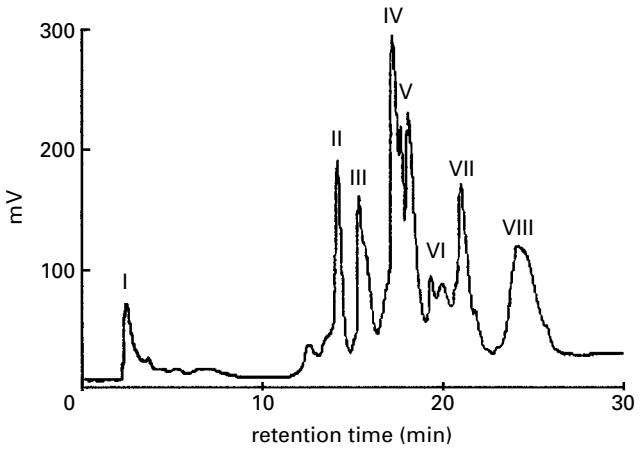

Fig. 3. The HPLC elution of OPN treated with pepsin. The chromatogram represents OPN incubated with pepsin at 37 °C for 24 h. The inhibitory activity of the constituents of the numbered fractions on the formation of calcium phosphate precipitates was estimated, and N-terminal amino acid sequence analysis was performed.

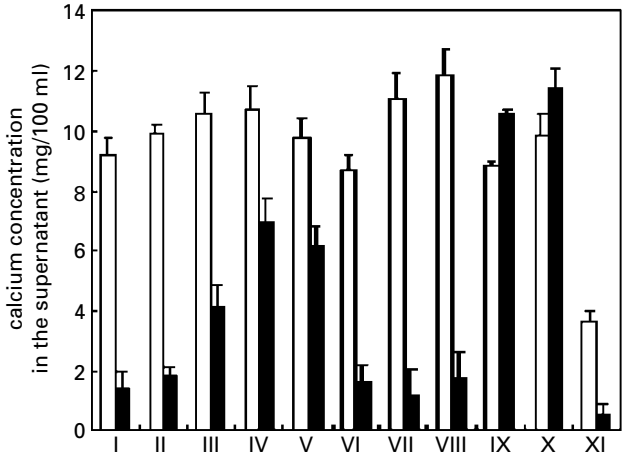

Fig. 4. Inhibitory activity of HPLC fractions in the formation calcium phosphate precipitates. Sodium phosphate solution containing fractions (I ~ VIII), obtained by HPLC as shown in Fig. 3 was mixed with calcium chloride solution and incubated for 3 h at room temperature. Calcium concentration in the soluble fraction of 0 h incubation (open bar) and 3 h incubation (closed bar) was determined. OPN incubated with pepsin at 37 °C for 24 h (IX), intact OPN (X) and a control that contained neither OPN nor OPN digest (XI) were also evaluated. The error bar represents standard error.

(32 μg/ml), the inhibitory effect was detectable in the initial stage of incubation. The results of single or dual digestion of OPN by trypsin and/or pepsin were similar to that of intact OPN (data not shown), which implied that the inhibitory effect on the formation of calcium phosphate precipitates was dependent rather on local amino acid sequence than on the structural conformation of OPN. Since pepsin proved to be more suitable for obtaining OPN fragments (Fig. 1), OPN was treated with pepsin for 24 h to recover eight fractions by HPLC (Fig. 3). Three fractions, peaks III, IV and V, exhibited inhibitory activity (Fig. 4). The results of N-terminal amino acid sequence analysis demonstrated that the presence of Y_{142}-(YGLKSRS……) in the peak III with negligible amount of contaminants. In the peak IV, the peptide of L_1-K_4 (LPVK) dominated, with slight contamination by the peptide Y_{142}-. Although peak V contained multiple peptides, W_{27}-(WLKPD……) was the only sequence that matched that of bovine OPN. Subsequently, we synthesized two peptides of L_1-K_4 (LPVK) and Y_{142}-R_{152} (YGLKSRSKKFR); the latter was synthesized on the basis of results of MALDI-TOF-MS. However, no synthetic peptide exhibited inhibitory activity, either as a single peptide at a concentration ranging from 10 to 100 μg/ml or in peptide cocktails (50 μg/ml each).

Discussion

The purpose of this study was to confirm the ability of bovine milk OPN and its fragments to prevent formation of calcium phosphate precipitates, and to compare its activity with that of CPP. The activity of interest after OPN was treated with intestinal proteases was comparable to that prior to digestion and higher than that of CPP.

It has been shown that phosphorylation of OPN or its peptides was required to inhibit formation of hydroxyapatite (Hunter et al. 1994; Jono et al. 2000; Pampena et al. 2004). Since phosphorylated amino acid residues are distributed throughout the OPN molecule, subsequent

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attention was aimed at identifying the crucial phosphorylated peptide(s), responsible for the activity. The HPLC fractions with peaks III, IV and V (Fig. 3) exhibited the activity of interest, however, neither synthetic peptide exhibited the activity of interest. This inconsistent result was in part due to inappropriate interpretation of the results of MALDI TOF-MASS analysis, which led to underestimation of the molecular mass of the genuine peptide. We could not exclude the possibility of the presence of the peptide with a phosphorylated amino acid residue ranging from Y_{142} to a residue more proximal to C-terminal than R_{152}, which was found in peak III and IV. It was of interest to note the structural features of this peptide, in which basic amino acids, such as lysine and arginine, were exclusively concentrated in N-terminus whereas acidic amino acids, such as glutamic acid and aspartic acid, were located in distal region. Although phosphorylated amino acid residues were less frequently located in this region (Sørensen et al. 1995), such electrostatic distribution might be suitable for the activity of interest. The highest level of inhibition was monitored in the peak IV, in which a peptide of L_1-K_4 was dominated. The disappearance of inhibitory activity using the synthetic peptide of L_1-K_4 implied a crucial structural feature for inhibitory activity, which is lacking in synthetic peptide of L_1-K_4. Structural comparison of the peptide of L_1-K_4 released from OPN to that of synthetic copy would be needed to explain this question. Additional information will be available when the peptide in the peak V, responsible for this inhibitory activity is successfully identified by further purification.

Our previous report demonstrated that chymosin cleaved OPN at the sites of I_{26}-W_{27} and R_{152}-R_{153} at pH 6·2 (Kumura et al. 2004), which indicated the presence of common cleavage sites for pepsin and chymosin. It was interesting to note that the susceptibility of OPN to chymosin was maximal at pH 4·0, where β-lactoglobulin was resistant to chymosin (data not shown). Consequently, OPN fragments could be prepared from cheese whey only if the whey would be set to allow growth of the residual starter lactic acid bacteria to reach the pH 4·0 where, maximum proteolytic action of chymosin would be exerted and the resulting OPN peptides could be easily isolated using ultra-filtration or some other industrial technology.

In summary, we demonstrated that the inhibitory activity of OPN on the formation of calcium phosphate was superior to that of CPP. It was suggested that one of the regions responsible for this activity was situated in the center of the OPN molecule. Since the inhibitory activity was maintained even after dual treatments with pepsin and trypsin, it would be of interest to conduct an *in vivo* study to determine whether oral administration of OPN could influence calcium absorption efficiency as CPP has been shown to promote calcium availability (Lee et al. 1992; Saito et al. 1998). More attention should be paid to physiological significance of ingested OPN because OPN in human milk is abundant compared with that in bovine mammary secretion. Such studies could provide new information not only to develop novel functional food ingredients, but also to improve the quality of infant formulas.

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