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Susceptibility of bovine osteopontin to chymosin

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Osteopontin (OPN) is an acidic phosphorylated glycoprotein found in many tissues and physiological fluids. Bovine OPN is a mature protein comprising 262 amino acids with a calculated molecular weight of 29 kDa. However, SDS-PAGE analysis reveals that the protein isolated from milk migrates to a molecular mass of 60 kDa (Sørensen & Petersen, 1993; Bayless et al. 1997). Bovine milk OPN is phosphorylated at 27 serine residues and one threonine residue (Sorensen et al. 1995); three O-glycosylated threonines were also identified, but no asparagine residues were glycosylated in spite of the presence of three putative N-glycosylation sites. In contrast, eight phosphates are recognized in bovine bone OPN (Salih et al. 1996), and 12 phosphoserines and one phosphothreonine are proposed in addition to five O-linked oligosaccharides and at most one N-linked oligosaccharide in the case of rat bone OPN (Prince et al. 1987). Thus, the possibility of tissue or species-specific differences in post-translational modification has been discussed.

A number of reports discuss the function of OPN, including cell adhesion, calcium remodelling, cell signalling and cellular transformation, and these functions have been extensively reviewed (Butler et al. 1996; Sodek et al. 2000). In mammary gland, high levels of OPN expression are observed in early lactation and involution (Rittling & Novick, 1997). Nemir et al. (2000) describe the biological significance of OPN in mammary gland differentiation. It is claimed that OPN is more abundant in colostrum (Uede et al. 1997), which contains relatively high levels of proteins that confer immunological defence in newborns, such as lactoferrin, lactoperoxidase and immunoglobulins. However, little attention has been paid to the biological significance of OPN in the milk supplied to newborns. In addition, we lack fundamental information on the concentration and molecular properties of OPN, which is necessary to determine its possible roles in mammary secretions.

In this study, we used immunodetection to measure OPN in bovine mammary secretions during early lactation. Furthermore, because it appears exclusively in the

abomasum of newborn ruminants, we examined the susceptibility of OPN to chymosin. In addition, some molecular properties including deletion and glycosylation are also discussed.

Materials and Methods

Samples

Samples of colostrum and milk were obtained individually from the first 10 milkings *post partum* (days 1–5 of lactation) from seven Holstein-Friesian cows from the Hokkaido University Experimental Farm. Colostrum was obtained immediately after parturition. The cows were milked twice daily. Purification of OPN was carried out according to the procedure of Maeda et al. (2002) using fresh raw milk obtained from the Holstein herd at the experimental farm. In brief, raw milk was skimmed by centrifugation and the resulting skim milk was acidified to pH 4.6 using 1 M-HCl. The precipitates were removed by centrifugation to obtain acid whey followed by addition of 1 M-NaOH to adjust pH to 5.0. The acid whey was applied to DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M-acetate buffer pH 5.0. After washing off unadsorbed materials, elution was carried out by linear gradient of NaCl dissolved in the same buffer. The fraction containing OPN was pooled and dialysed against 0.1 M-acetate buffer pH 5.0. Re-chromatography was carried out using DEAE-Toyopearl 650S (Tosoh, Tokyo, Japan) equilibrated with the same buffer, followed by elution with linear gradient of NaCl dissolved in the same buffer.

Protein determination

Protein concentration was determined by a modified Lowry method (Bensadoun & Weinstein, 1976) using bovine serum albumin as a standard.

Polyclonal antibodies

Two polyclonal antibodies for human OPN (hOPN2 and hOPN4) were used (Kon et al. 2000).

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Electrophoresis and immunoblotting

Samples were loaded on to a 12.5% polyacrylamide gel containing 0.1% SDS according to the procedure of Laemmli (1970). 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, NY, USA) according to the procedure of Green et al. (1973).

For immunoblotting analysis, proteins in the gel were electrophoretically transferred to a PVDF membrane (Osmonics, Westborough, MA, USA.) using a semi-dry system (Tanaka et al. 1999). The membrane was blocked by soaking in a solution of 1% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS) and the membrane was incubated with hOPN2 or hOPN4 dissolved in 1% BSA/PBS solution. Signals were detected using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and chemiluminescence with CDP-Star.

Molecular properties of OPN against some enzymes

Purified OPN was incubated with glycosidases or chymosin. Ten micrograms of the purified OPN was boiled for 5 min in the presence of 1% SDS and diluted 20-fold with 20 mM-sodium phosphate buffer (pH 7.2) containing 0.05% Triton X-100. The resulting mixture was incubated at 37 °C for 18 h in the presence of 2 U of N-glycosidase F or 2.5 mU of O-glycosidase supplemented with 5 mU of sialidase. These enzymes were obtained from Roche (Mannheim, D-68305 Germany).

Sensitivity against calf chymosin was investigated by incubation of the purified OPN (0.25 mg) dissolved in 1 ml of 20 mM-PIPES (pH 6.2) containing 1 mM-CaCl₂ in the presence of defined units (0.01 to 1.0 U) of commercial recombinant chymosin known as MAXIREN (Gist-brocades, Seclin, 59472, France). In our study, rennet activity was determined by the procedure of Berridge (1945). The reaction was performed at 37 °C for 3 h and terminated by the addition of 1% SDS-containing buffer prepared for electrophoresis.

Isolation of fragments released from OPN by chymosin

Purified OPN treated with 0.1 U of MAXIREN at 37 °C for 3, 8 or 24 h was loaded on a CAP CELL PAK C8 column (4.6 × 150 mm; Shiseido Co., Ltd., Ginza, Japan). Alternatively, the MAXIREN-treated sample was fractionated using a centrifugal filter of Ultrafree-MC tube (Millipore, Bedford, MA 01730, USA) with a molecular weight cut-off of 10 kDa, at 1800 g at 4 °C for 30 min to obtain a filtrate. Experimental conditions were as previously described (Kumura et al. 1999).

N-terminal amino acid sequence analysis

N-terminal amino acid sequence of the purified OPN and the fractions obtained by HPLC was determined using a

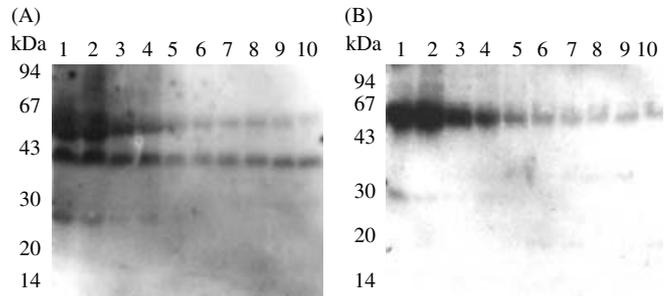


Fig. 1. Western blotting of mammary secretions during early lactation. Two microlitres of ten-fold diluted sample was loaded on each lane for SDS-PAGE and detected with hOPN2 (A) or hOPN4 (B). Lane 1, just after parturition (day 1 morning); lane 2, day 1 evening; lane 3, day 2 morning; lane 4, day 2 evening; lane 5, day 3 morning; lane 6, day 3 evening; lane 7, day 4 morning; lane 8, day 4 evening; lane 9, day 5 morning; lane 10, day 5 evening.

protein sequencer (model 492A, Applied Biosystems, Foster City, CA, USA).

Results and Discussion

Results obtained by Western blot analysis using one representative sample of individual bovine mammary secretions taken during the first 10 milkings are shown in Fig. 1. Two distinct bands of 60 and 40 kDa were recognized by the antibody hOPN2 (Fig. 1A). In contrast, a band of 60 kDa was found when hOPN4 was used (Fig. 1B). The signal from the 60-kDa band exhibited the same progressive decrease of intensity over time when using hOPN2 or hOPN4. Slight signals of 26 kDa and 28 kDa were detected using hOPN2 and hOPN4, respectively. However, these signals appeared less frequently and varied between individual cows.

To identify the 40-kDa signal detected by hOPN2, purification of OPN from bulk milk was performed. Analysis of the N-terminal amino acid sequence of purified OPN gave LPVKP, indicating that the OPN preparation was highly purified and contained negligible amounts of contaminants. Nevertheless, both signals of 60 and 40 kDa were recognized when the purified OPN was analysed by Western blotting with hOPN2 (Fig. 2B, lane 3 and 4). It is interesting to note that hOPN2 was established using N₁₈-Q₃₄ as the epitopic region, whilst hOPN4 was prepared using K₂₄₄-N₂₆₂ as the epitopic region, which is located in the last C-terminal region (Kon et al. 2000). The N-terminal sequence analysis of the purified OPN gave the result expected for intact mature bovine OPN. Therefore, it can be concluded that the signal observed at 40 kDa was the result of a deletion of the C-terminal region of OPN. In human milk, a component of 35 kDa appears in addition to intact OPN, which had a molecular mass of 75 kDa (Senger et al. 1989). This 35-kDa component, predicted as the product of natural cleavage of OPN, may be analogous

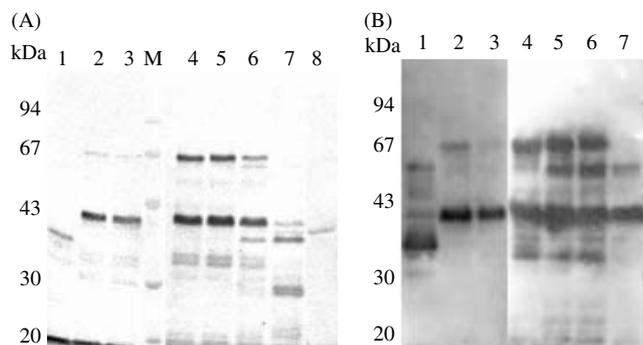


Fig. 2. Susceptibility of OPN to glycosidases and chymosin. Samples were loaded on each lane for SDS-PAGE and stained with Stains-All (A) or detected by Western blotting using hOPN2 (B). OPN incubated with O-glycosidase and sialidase (lane 1), OPN incubated with N-glycosidase F (lane 2), purified OPN (lane 3 and 4), OPN incubated with 0.01 U of chymosin (lane 5), OPN incubated with 0.1 U of chymosin (lane 6), OPN incubated with 1 U of chymosin (lane 7), 1 U of chymosin (lane 8). M=molecular mass marker comprising phosphorylase b (97 kDa), Albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20 kDa).

to the 40-kDa band observed in the present study. However, when proteins from rodent mammary gland tissue after 2 d of lactation are extracted and analysed, the corresponding polypeptide from OPN is not detected by Western blot analysis (Rittling & Novick, 1997). This suggests that generation of the 35–40 kDa derivative in mammary secretions depends on the animal species. In this study, the C-terminal counterpart was undetectable, which was attributed to its high susceptibility to proteolysis.

Further characterization of bovine OPN was performed with regard to glycosylation. The electrophoretic mobilities of both the 60- and 40-kDa OPN were unaffected by incubation of OPN with N-glycosidase (Fig. 2A and 2B, lane 2), whereas the molecular mass of these OPN decreased by 5–8 kDa when O-glycosylation site(s) were eliminated (Fig. 2A and 2B, lane 1). Sørensen et al. (1995) report the presence of three O-glycosylated threonines (Thr₁₁₅, Thr₁₂₄ and Thr₁₂₉) and no N-glycosylation site in bovine milk OPN, despite the presence of three putative N-glycosylation sites. Thus, our result demonstrated that three O-glycosylated threonines were retained in the 40-kDa OPN, which possessed more than 129 amino acid residues in the N-terminal region of mature bovine OPN. Since the molecular mass of bovine OPN without any post-translational modification was predicted to be 29 kDa, the electrophoretic mobility of the glycosylated OPN should have been 35 kDa. However, the electrophoretic mobility was revealed to be slower than expected and this was attributed to the high degree of phosphorylation of OPN.

The biological significance of intact or partially digested OPN in milk has yet to be elucidated. The fact that OPN is abundant in colostrum and that chymosin appears

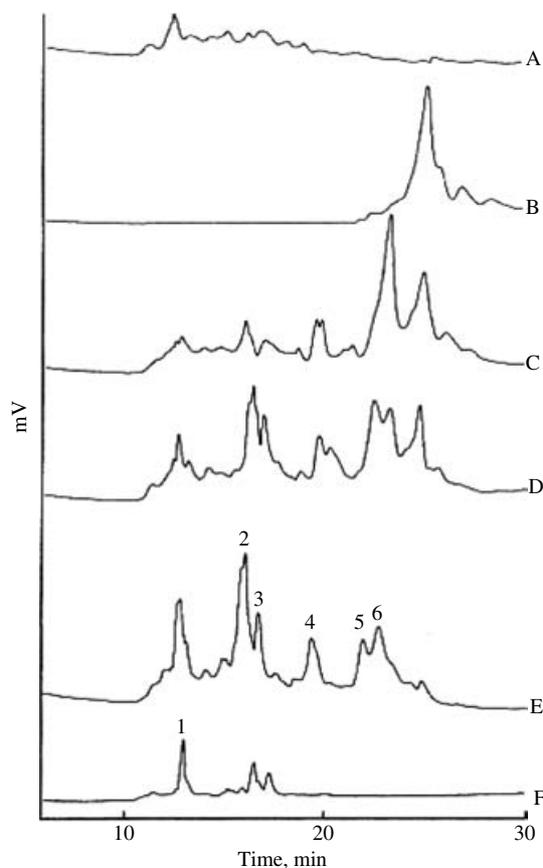


Fig. 3. HPLC elution of OPN treated with chymosin. Chromatogram represents chymosin (A), intact OPN (B), OPN incubated with 0.1 U of chymosin at 37 °C for 3 h (C), for 8 h (D), for 24 h (E) and 24 h followed by fractionation using centrifugal filter (F). The constituents of the numbered fractions were subjected to N-terminal amino acid sequence analysis.

exclusively in the abomasum of newborn ruminants led us to speculate that the liberation of biologically active fragments from OPN could be induced by chymosin. When OPN was incubated with 0.01 U of chymosin, faint bands corresponding to molecular masses of 57 kDa and 37 kDa appeared (Fig. 2A, lane 5), which was more pronounced when OPN was incubated with 0.1 U of chymosin (Fig. 2A, lane 6). Deletion of 3 kDa peptide may have occurred as the primary event in both the 60- and 40-kDa form of OPN. Increasing proteolytic activity up to 1 U led to the appearance of polypeptides of 28 and 29 kDa in addition to accumulation of one of 37 kDa (Fig. 2A, lane 7). These resulting fragments were shown to lack the N-terminal region of intact OPN because they were undetectable by Western blotting using hOPN2 (Fig. 2B, lane 5–7). Conversely, 0.01 U of chymosin released a polypeptide of 50 kDa, which was poorly stained with Stains-All, but responsive to hOPN2 (Fig. 2A and 2B, lane 5). According to Foltmann (1971) 8.9×10^5 rennet units are obtained from 28 dried calf stomachs. Simply calculated, 3.1×10^4 rennet units could be present in a single

stomach. Therefore, it is conceivable that extensive degradation of colostrum OPN would occur by the action of chymosin.

To identify cleavage sites, fractionation of fragments was attempted using HPLC. In this experiment, the amount of chymosin was reduced to 0.1 U in order to facilitate resolution of chymosin and, conversely, the incubation period was extended up to 24 h. As shown in Figs 3B–3E, OPN was progressively digested and finally, multiple peaks were recognized (Fig. 3E). The peak of earlier retention time seemed to contain not only OPN fragment but also chymosin by comparison with the major peak of MAXIREN (Fig. 3A). Therefore, before applying it to the HPLC column, the sample was fractionated using a centrifugal filter whose cut-off molecular mass was 10 kDa. In the resulting filtrate, three peaks were found in the earlier retention time (Fig. 3F). Finally, we collected six numbered samples to determine the N-terminal acid sequence.

Determination of peak No. 1 (Fig. 3F) identified the cleavage site of I₂₆–W₂₇ and peak No. 4 (Fig. 3E) was predicted to arise from I₂₆–W₂₇ and R₁₅₂–R₁₅₃. In peaks No. 2 and 3, the N-terminal amino acid sequence of mature OPN was recognized. Peak No. 5 contained peptides considered as SQEF and DDDDD, which corresponded to cleavage sites of L₂₂₃–S₂₂₄ and L₇₂–D₇₃, respectively. The latter sequence was found in peak No. 6 as well. Differences of susceptibility of the dual forms of OPN to chymosin remains unclear. In any case, the cleavage profile was concluded to be indefinite. However, in terms of its location and molecular mass, it could be postulated that the site of I₂₆–W₂₇ was preferentially cleaved by chymosin to release 57-kDa and 37-kDa OPN fragments. In the C-terminal region, site(s) sensitive to chymosin existed because of the presence of a 50-kDa fragment, responsible for hOPN2, although the cleavage site remained unclear.

Senger et al. (1989) report OPN concentration in human milk of 3–10 mg/l. From 1 l of bovine milk, Sorensen and Petersen (1993) and Bayless et al. (1997) isolated approximately 22 mg and 8 mg of OPN, respectively. However, no information was available on the concentration of OPN in bovine colostrum or mature milk. Therefore we performed Western blotting of serial dilutions of seven colostrums and milks from the 10th milking *post partum* with reference to a known quantity of purified OPN. As a result, we measured <200 mg/l in colostrum and at day 5 of lactation, it had decreased to less than 10% of its initial level. Consequently, the concentration of OPN was much lower than that of immunoglobulins or lactoferrin (Scammell, 2001), but the abundance of OPN in colostrum was confirmed.

In conclusion, we demonstrated effects of chymosin on OPN and confirmed the presence of intact and partially degraded OPN in mammary secretions. Further investigations are needed to elucidate the biological significance to the newborn of OPN and/or the fragments released by chymosin.

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