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PRODUCTION OF MONOCLONAL ANTIBODIES TO PROSTROMELYSIN (ProMMP-3) AND ESTABLISHMENT OF A QUANTITATIVE PROSTROMELYSIN ELISA ASSAY

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Human prostromelysin (59 kDa) was purified from the conditioned medium of IL-1-stimulated human dermal fibroblasts and anti-prostromelysin monoclonal antibodies were produced and identified by ELISA assay. Using prostromelysin, a C-terminally truncated recombinant form of prostromelysin consisting of amino acids 1-255, and their respective activated enzymes, we have begun mapping the epitopes recognized by these monoclonal antibodies. Various patterns of reactivity against the proenzymes and activated enzymes were observed. In further attempts to map the epitopes, we employed synthetic peptides representing hydrophilic regions of the primary amino acid sequence of prostromelysin. Our monoclonal antibodies did not recognize these peptides, suggesting that the antibodies may be recognizing conformational epitopes composed of non-linear portions of prostromelysin. Using these monoclonal antibodies, we have developed a quantitative prostromelysin sandwich ELISA assay.

Stromelysin (MMP-3) is a matrix metalloproteinase which plays an important role in the degradation of extracellular matrix components including proteoglycans and collagen (1). In addition, stromelysin has been implicated in the activation of interstitial procollagenase to collagenase in the extracellular fluid (2). Recent reports demonstrating elevated levels of stromelysin activity in the joints of patients with osteo and rheumatoid arthritis (3,4) have led to interest in the regulation of prostromelysin expression under various pathophysiological states. Here we describe the production of prostromelysin-specific monoclonal antibodies, and their use in establishing a sensitive, specific sandwich ELISA which can be used to measure prostromelysin levels in physiologic fluids.

Materials and Methods

Polyclonal Antibodies

Polyclonal antibodies against human fibroblast stromelysin were raised in sheep. Purified prostromelysin, kindly provided by Dr. H. Nagase (Univ. of Kansas Medical Center, Kansas City,

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KS) was activated with (4-aminophenyl)mercuric acetate (5), dialyzed, and mixed with Freund's complete adjuvant. This emulsion, containing 100 μg of stromelysin, was injected intradermally at multiple sites on the sheep. Subcutaneous booster injections of 50 μg of stromelysin in Freund's incomplete adjuvant were performed, and the sheep was bled 14 days after each boost. The IgG fraction was purified from serum as described (6).

Purification of Prostromelysin, Recombinant C-Terminal Truncated Prostromelysin, and Synthetic Prostromelysin Peptides

Prostromelysin was purified from the culture medium of IL-1-stimulated human dermal fibroblasts using sheep anti-stromelysin IgG coupled to Affi-Gel 10 (Bio-Rad, Richmond CA) as previously described (5). The C-terminal truncated form of human fibroblast prostromelysin was expressed in *E. coli* and purified to homogeneity as described by Marcy *et al.* (7). This protein, when activated by (4-aminophenyl)mercuric acetate, consists of amino acids 83-255 of full-length stromelysin and has catalytic activity equivalent to the native protein (7). SDS-PAGE was performed according to the method of Laemmli (8). Synthetic peptides ranging in size from 12-27 amino acids representing hydrophilic regions of the primary amino acid sequence of prostromelysin were synthesized on a 431A Peptide Synthesizer (Applied Biosystems Inc., Foster City, CA) and purified by HPLC. The final products were characterized by fast atom bombardment mass spectrometry, amino acid analysis, and analytical HPLC.

Generation of Anti-Prostromelysin Monoclonal Antibodies

Female Balb/c mice were immunized intraperitoneally (i.p.) with 20 μg purified prostromelysin in Freund's complete adjuvant. The mice were boosted i.p. with 20 μg prostromelysin in Freund's incomplete adjuvant on days 24 and 45, and serum anti-prostromelysin activity was assessed by ELISA assay. Following three daily boosts with 10 μg prostromelysin in PBS, the spleen was harvested and the splenocytes were fused with SP2/0 murine myeloma cells using standard procedures (9). Hybridomas were grown in RPMI 1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT), 5% P388D1 conditioned medium, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, and HAT medium supplement (Sigma, St. Louis, MO). Hybridoma supernatants were tested for reactivity with prostromelysin by ELISA assay. All positive hybridomas were subcloned by limiting dilution.

ELISA Assay

Hybridoma supernatants or purified antibodies were incubated in prostromelysin-coated wells, the wells were washed with PBS containing 0.05% Tween 20, and peroxidase-conjugated γ-specific goat anti-mouse IgG (Caltag, So. San Francisco, CA) was added to the wells. After washing with PBS containing 0.05% Tween 20, TMB substrate solution (Kirkegaard and Perry, Gaithersburg, MD) was added, and the absorbance at 450 nm was measured. For experiments examining synthetic peptides, the peptides were coated at a concentration of 1 μg/ml in 0.1 M sodium carbonate buffer, pH 9.6 and were allowed to dry overnight onto the plates.

Western Blots

Unreduced samples (15 μg) of the indicated form of stromelysin were heated at 37°C for 3 min in Laemmli sample buffer (8) containing 2% SDS. The proteins were run on 4-20% gradient acrylamide minigels (Bio-Rad) using a preparative sample comb. After SDS-PAGE, the proteins were transferred to Zeta-Probe blotting membranes (Bio-Rad) and blocked in 5% non-fat milk solution. The membranes were then placed in a Miniblotter 28 (Immunetics, Cambridge, MA), and were incubated with 50 μl of the indicated antibodies at 5 μg/ml in 1% BSA in 0.5 M NaCl, 0.02 M $NaPO_4$, 0.05% Tween 20, pH 6.5. The blots were treated with 5 μg/ml rabbit anti-mouse IgG (Cappel, Durham, NC), washed, incubated with goat anti-rabbit IgG-HRP (Bio-Rad) and developed with the ECL Detection System (Amersham, Arlington Heights, IL).

Prostromelysin Sandwich ELISA Assay

Immulon II plates (Dynatech, Chantilly, VA) were coated with 5 μg/ml 10B6 in 50 mM sodium carbonate buffer, pH 9.6 and then blocked with 3% BSA in PBS. Aliquots (50 μl) of the indicated concentration of prostromelysin diluted in 1% BSA in 0.5 M NaCl, 0.02 M $NaPO_4$, 0.05% Tween 20, pH 6.5 were added to the wells, incubated overnight at 4°C, and the wells were washed with PBS containing 0.05% Tween 20. Captured prostromelysin was detected using biotinylated 19C5 (5 μg/ml) followed by Vectastain ABC Kit (Vector Labs, Burlingame, CA) or sheep anti-stromelysin IgG (5 μg/ml) followed by donkey anti-sheep IgG-HRP (Jackson Immunoresearch Laboratories, West Grove, PA). The assay was developed using TMB substrate kit (Kirkegaard and Perry), and the absorbance at 450 nm determined.

Results

Purified Forms of Prostromelysin

Figure 1 shows the purity of the various proteins used in this study. Human fibroblast prostromelysin consists of two major protein stained bands at 57 and 59 kDa representing non-glycosylated and glycosylated forms of the protein, respectively. The minor band at 47 kDa represents a small amount of active stromelysin in the protein preparation. Upon treatment of prostromelysin with (4-aminophenyl)mercuric acetate, prostromelysin is converted to two major species with molecular weights of approximately 47 and 49 kDa. These results are similar to those reported by Nagase *et al.* (10). Purified truncated prostromelysin and (4-

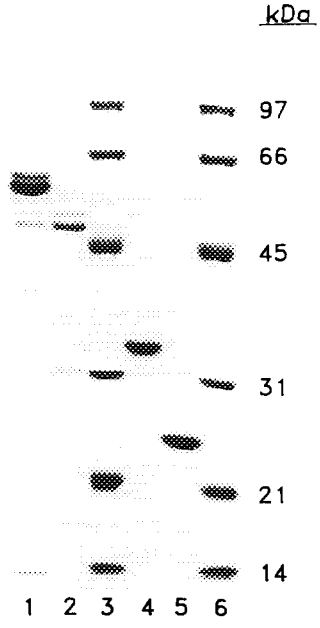

Figure 1. SDS-PAGE (10% Gel) of Purified Pro and Activated Stromelysin. Lanes 1 and 2, 3 μg of human fibroblast prostromelysin and stromelysin, respectively. Lanes 4 and 5, 2 μg of recombinant truncated human prostromelysin and stromelysin, respectively. Lanes 3 and 6, molecular weight standards.

Table 1. Reactivity of monoclonal antibodies against full-length and C-terminal truncated pro and active stromelysin in ELISA assay

	Full-Length		C-Terminal Truncated	
	Proenzyme	Activated	Proenzyme	Activated
10D12	+	+	+	+
25H6	+	+	+	+
5C4	+	-	+	-
11D10	+	-	+	-
24G6	+	-	+	-
7G3	+	+	-	-
11B7	+	+	-	-
12F12	+	+	-	-
16F11	+	+	-	-
24G8	+	+	-	-
25H4	+	+	-	-
5D6	+	-	-	-
5D12	+	-	-	-
10B6	+	-	-	-
13D7	+	-	-	-
16H9	+	-	-	-
17E2	+	-	-	-
19C5	+	-	-	-

aminophenyl)mercuric acetate activated stromelysin have apparent molecular weights of 33 and 24 kDa, respectively, in agreement with the previously reported results (7).

Monoclonal Antibodies Against Prostromelysin

Monoclonal antibodies that recognize prostromelysin in an ELISA assay were identified and subcloned. In an effort to map the epitopes recognized by these antibodies, the reactivity of these antibodies in ELISA and Western blot experiments employing various forms of stromelysin was determined. Table 1 summarizes the reactivity of our monoclonal antibodies against prostromelysin, C-terminal truncated prostromelysin, and their respective activated enzymes when coated on ELISA plates. The monoclonal antibodies displayed different patterns of reactivity with the various forms of stromelysin, as grouped in Table 1. Western blot analysis, shown in Figure 2 and summarized in Table 2, gave similar results, although discrepancies did exist between reactivity in ELISA and Western blots for some of the antibodies. To further map the epitopes recognized by these antibodies, synthetic prostromelysin peptides representing amino acid regions 1-14, 26-49, 63-88, 79-90, 107-126, 134-143, 144-152, 177-192, 196-213, 228-254, 261-276, 285-297, 298-314, 322-333, 346-357, 366-382, 388-403, 406-425, and 444-460 were synthesized. None of these peptides were recognized by any of our anti-prostromelysin monoclonal antibodies, although anti-peptide antisera did recognize these peptides in our ELISA format (data not shown), suggesting that non-linear conformational epitopes were being recognized by the antibodies.

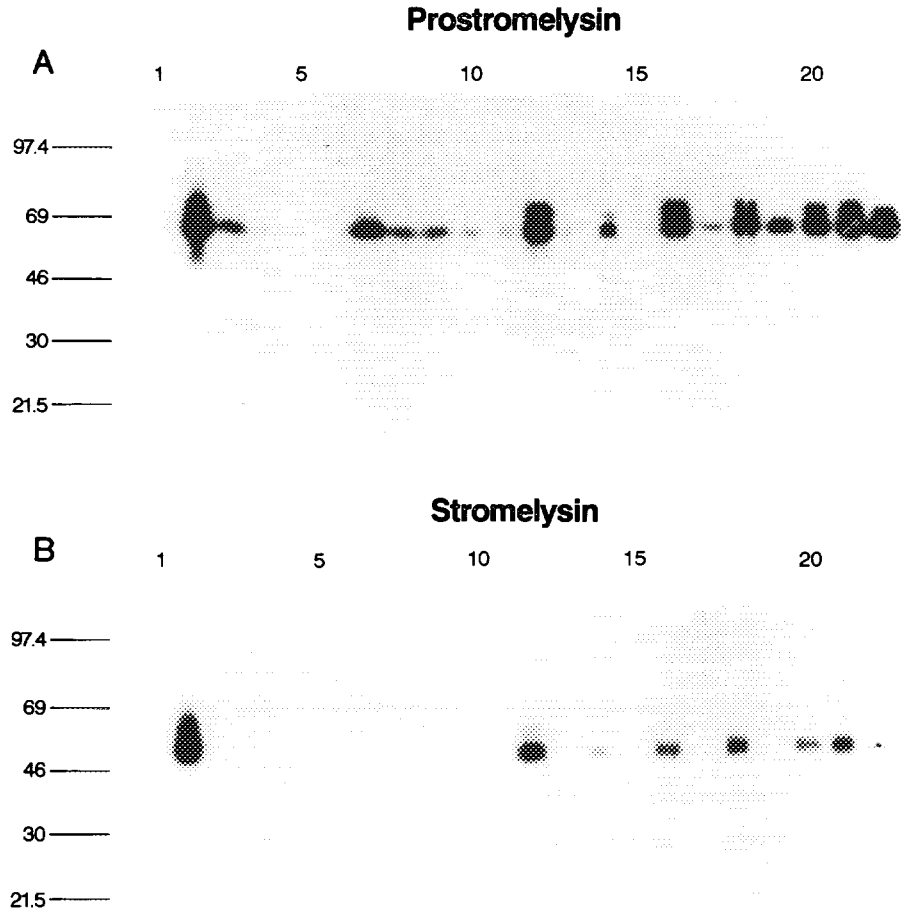

Figure 2. Western Blot Analysis of Anti-Prostromelysin Monoclonal Antibodies. Antibody reactivity against pro and active full-length stromelysin is shown. Table 2 identifies the monoclonal antibody in each lane and summarizes all the Western Blot data.

Quantitative Prostromelysin Sandwich ELISA Assay

Using various combinations of monoclonal and polyclonal anti-prostromelysin antibodies, we have developed a quantitative sandwich ELISA for prostromelysin. Our antibody 10B6 proved to be the best at capturing prostromelysin onto the ELISA plate. Captured prostromelysin could be detected either with a sheep polyclonal antiserum, or with biotinylated monoclonal antibody 19C5. Typical standard curves for these assays are shown in Figure 3. The sensitivity of the assay was 5 ng prostromelysin/ml. Neither assay recognized activated stromelysin, as expected since capturing antibody 10B6 is prostromelysin-specific, or collagenase (MMP-1) (Figure 3). In addition, 500 ng/ml gelatinase B (MMP-9), vitronectin, laminin, fibronectin, and collagen type IV did not react in this assay.

Table 2. Reactivity of monoclonal antibodies against full-length and C-terminal truncated pro and active stromelysin in Western Blots. Serum from the fusion mouse was included as a positive control.

		Full-Length		C-Terminal Truncated	
Lane #		Proenzyme	Activated	Proenzyme	Activated
1	Buffer	-	-	-	-
2	Serum (1:1000)	+	+	+	+
3	5C4	+	-	+	-
4	5D6	+	-	-	-
5	5D12	+	-	-	-
6	Neg. Control	-	-	-	-
7	7G3	+	+	+	+
8	8G8	+	+	-	-
9	10B6	+	-	-	-
10	10D12	+	-	-	-
11	11B7	+	+	-	-
12	11D10	+	+	-	-
13	12F12	+	-	-	-
14	13D7	+	+	-	-
15	16F11	+	-	-	-
16	16H9	+	+	-	-
17	17E2	+	-	-	-
18	19C5	+	+	-	-
19	24G6	+	-	+	-
20	24G8	+	+	-	-
21	25H4	+	+	-	-
22	25H6	+	+	+	+

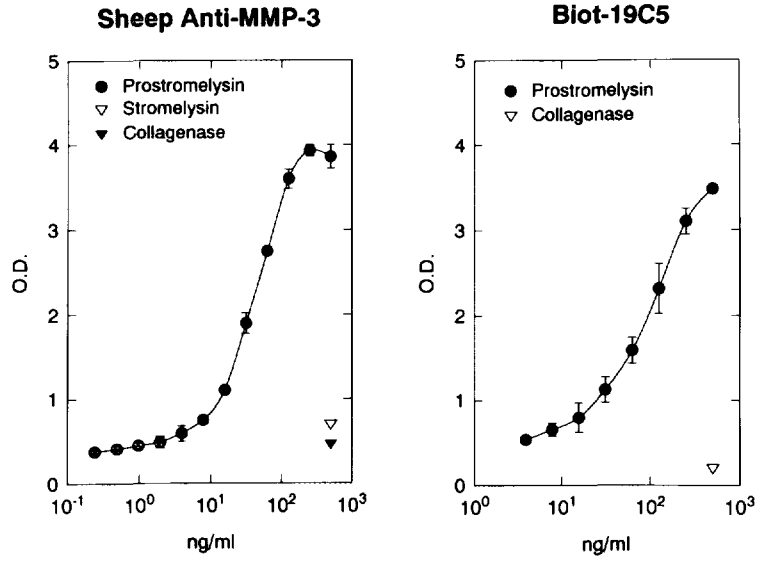

Figure 3. Sandwich ELISA for Prostromelysin. Prostromelysin at the indicated concentration was incubated in plates coated with monoclonal antibody 10B6. Captured prostromelysin was detected with the indicated secondary reagent.

Discussion

We have described the production of a panel of anti-prostromelysin monoclonal antibodies with differential reactivity to various forms of stromelysin. Using these antibodies, we have established a specific, sensitive sandwich ELISA assay for prostromelysin. The assay did not exhibit cross-reactivity to other matrix metalloproteinases or extracellular proteins. In contrast to a previously reported stromelysin assay (11), the sandwich ELISA described here did not exhibit reactivity with activated stromelysin. This ability to selectively measure prostromelysin protein levels will aid in the study of the regulation of prostromelysin expression.

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