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Assay of collagenase activity for native triple-helical collagen using capillary gel electrophoresis with laser-induced fluorescence detection

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Abstract

Three collagenase assays for two native triple-helical collagens have been developed using capillary gel electrophoresis (CGE) with laser-induced fluorescence (LIF) detection in order to discover the matrix metalloproteinases (MMPs) inhibitors. These collagenase assays include measurement of the activities of interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8) against type I collagen, and collagenase-3 (MMP-13) against type II collagen, and the enzyme activities could be readily measured by determining the 3/4 fragments produced from the cleavage of the native collagens. The highly desired sensitivity of the assays could be achieved, employing a dynamic fluorescence labeling technique with the running buffer containing 0.05% sodium dodecylsulfate and non-covalent fluorescent dye for protein, NanoOrange. The collagen, its 1/4 and 3/4 fragments of type I or II collagen could be separated and detected within the run time of 20 min by CGE mode using the gel buffer (pH 8.8) containing 4% polyacrylamide. Good linearity of the peak area of the 3/4 fragment was obtained over each assay range of collagenase (15–150 ng/tube for MMP-1, 3–30 ng/tube for MMP-8, and 1.5–30 ng/tube for MMP-13, respectively). The relative standard deviation of the peak areas of the 3/4 fragment produced from type II collagen by MMP-13 cleavage was calculated to be less than 13.4%, indicating that the assay was reproducible. Also, IC_{50} values of three MMPs inhibitors, which were calculated for estimation by the variation of the peak areas of the 3/4 fragments using 90 ng/tube for MMP-1, 30 ng/tube for MMP-8 or 15 ng/tube for MMP-13, were almost consistent with data from other assays. The CGE-LIF method is expected to be very useful for proteinase assay and its application to the estimation of inhibitors because this method enables an assay of collagenase activity using native substrate to be conducted without experimentally troublesome procedure such as preparation of antibody or fluorescence-labeled substrate.
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1. Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that degrade all of the major components of the extracellular matrix. The vertebrate collagenases are a group of MMPs distinguished by their capacity to cleavage triple-helical collagen at a single site resulting in fragments corresponding to 1/4 and 3/4 of its initial length. Three collagenases, interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13), have been already identified on the basis of sequence similarities and their ability to hydrolyze the fibrillar collagens (types I–III) [1]. Over-expression and activation of MMPs have been linked with a range of diseases for which good therapeutic approaches are currently sought, such as arthritis, cancer and multiple sclerosis [1–3]. In particular, the expression of MMP-13 and its osteoarthritic cartilage and its activity against type II collagen suggest that the enzyme plays a significant role in cartridge collagen degradation, and must consequently form part of a complex target for proposed therapeutic interventions based on collagenase inhibition [4]. Various inhibitors have been studied for the therapy of diseases linked with MMPs [1–3].

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A MMPs assay has been developed for the discovery of inhibitors, and the assay would be useful for high-throughput screening (HTS) at the first stage of drug discovery due to its advantages of high sensitivity, high speed and automation [5]. The assay using the coumarin-labeled peptide substrate has been in popular use for HTS; however, the other estimation of inhibitors should be needed to confirm the inhibition of collagenase activity using the native substrate, because the labeled peptide substrate has different characteristics from the native one. At the second stage, the drug candidates that have passed through the first stage need to be checked for whether they can inhibit the cleavage of triple-helical collagen by collagenase, using sodium dodecylsulfate-polyacrylamide gel electrophoresis [6], zymography [7–9] and collagenolysis assay [10–12] with native, fluorescence-labeled or radioisotope-labeled collagen as a substrate. However, these assays have drawbacks such as troublesome procedure and poor quantification. Therefore, it would be important to develop a convenient assay under desirable physical conditions using the native triple-helical collagen.

Capillary electrophoresis (CE) is a convenient analysis tool offering the advantages of high separation and efficiency, as reported elsewhere [13–15]. In addition, the array system based on CE is applicable to multiple assays, such as DNA sequencing [16] and enzyme assay [17] using the CE array system. Also, application of μ-chip electrophoresis and μ-TAS technique allows scaling down of the assay system and rapid measurement [18–20]. As the sensitivity of laser-induced fluorescence (LIF) detection is thought to be higher than that of ultraviolet detection, the CE-LIF method is useful as a highly sensitive enzyme assay, leading to a reduction in the amount of enzyme consumed for the assay [21]. Furthermore, having smaller amounts of reagents in samples placed into the capillary would raise the robustness of the methods.

In this work, we developed methods using capillary gel electrophoresis (CGE) with LIF detection for collagenase assays to evaluate the activity of inhibitors for drug discovery. Our method involves measuring of the activities of MMP-1 and MMP-8 against type I collagen, and MMP-13 against type II collagen, respectively. The enzyme activities could be readily measured by determining the 3/4 fragments produced from the cleavage of the native collagens. Jin et al. reported the dynamic fluorescence labeling method with the sieving matrix containing a non-covalent fluorescent dye for protein, NanoOrange [22]. However, higher sensitivity is need to determine the reaction products of collagenase. The modification of sodium dodecylsulfate concentration in CGE gel buffer significantly improved the sensitivity of the detection and enabled the collagenase assay using native substrate by CGE-LIF detection. This optimization of dynamic labeling enabled the collagenase assay using native substrate by CGE-LIF detection. This method showed good repeatability and was useful to estimate the IC_{50} values of inhibitors.

2. Experimental

2.1. Reagents

Bovine type II collagen was obtained from Collagen Research Center (Tokyo, Japan). Rat tail type I collagen was obtained from BD Biosciences (San Jose, CA, USA). Recombinant human MMP-1, MMP-8 and MMP-13 were obtained from Genzyme-Techne (Cambridge, MA, USA). NanoOrange dye was purchased from Molecular Probes (Eugene, OR, USA). NanoOrange was used according to the product instructions; NanoOrange solution (500×) was added to the gel running buffer at 3× concentration. Mass markers Protein Test Mix was purchased from Beckman Coulter (Fullerton, CA). *p*-Aminophenylmercuic acid (APMA) was obtained from Sigma (St. Louis, MO, USA). Acrylamide of high grade for electrophoresis, 2-amino-2-methyl-1,3-propanediol (AMPD) and cacodylic acid (CACO) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Ammonium peroxodisulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Nacalai Tesque (Kyoto, Japan). BB-2516, METI-SAM and MMI-166 were synthesized in our laboratory.

2.2. Apparatus

A P/ACE 5510 capillary electrophoresis system equipped with an argon ion laser emitting 488 nm and an LIF detector (Beckman Coulter) was used. Fluorescence emitted by samples was filtered by a 590 nm band-pass filter (Melles Griot, CA, USA). An eCAP-coated capillary (Beckman Coulter) with 100 μm i.d. × 360 μm o.d. of 27 cm (20 cm to detector window) was assembled in the LIF cartridge format, to which was applied constant voltage (−8.1 kV) at 20 °C. The running gel buffer used was 4% polyacrylamide and 0.05% SDS in 50 mM AMPD–CACO buffer (pH 8.8) with NanoOrange (3×) which was added to the gel buffer immediately before an assay batch. Samples were injected by pressure for 5 s at 0.5 psi. Prior to injection, the capillary was washed with 1% SDS for 2 min, water for 1 min and the running gel buffer for 4 min at each run. The electropherographic data were analyzed with Beckman P/ACE Station Software (version 1.0). LS50B spectrofluorometer (Perkin-Elmer, MA, USA) was used to determine the fluorescence intensity of type II collagen in the solution containing SDS and NanoOrange.

2.3. Collagenase activity assay

MMP-1 was activated by APMA according to the instructions provided with each reagent and was stored at 4 °C after the activation. Three microliters of activated MMP-1 solution (5–50 μg/ml) was added to 25 μl of 0.1 M Tris–HCl buffer (pH 7.4) containing 0.1 M sodium chloride, 10 mM calcium chloride and 0.01% Brij35 and 17 μl of distilled water. Next, 5 μl of type I collagen solution (3.96 mg/ml) was added followed by incubation for 2 h. To the reaction mixture, 5 μl of

10% SDS was added to quench the MMP-1 reaction and denature the proteins, and the sample solutions were subjected to CGE-LIF detection. The peak areas of the 3/4 fragments, which were generated just before substrate collagen, were monitored as an indicator of MMP-1 activity. MMP-8 assay was conducted in a similar manner as above-described MMP-1 assay, except for the amount of enzyme and the incubation time. The amount of MMP-8 used was from 3 to 30 ng/tube. The reaction time was applied at 1 h. In the MMP-13 activity assay, type II collagen was employed as the substrate. The amount of MMP-13 used was from 1.5 to 30 ng/tube. The other conditions were the same as those for MMP-8.

2.4. Evaluation of inhibitors

Using 90 ng/tube of MMP-1, 30 ng/tube of MMP-8 or 15 ng/tube of MMP-13, the evaluations of three existing inhibitors were implemented. Inhibitor solutions were prepared for dissolution and dilution with DMSO. Before addition of the substrate collagen, 1 μl of inhibitor solution was added, and the reaction and CGE-LIF analysis were carried out as described above. IC_{50} values were determined by collagenase activity analyses in the presence of several concentrations of each inhibitor.

3. Results and discussion

3.1. Optimization of LIF detection for collagenase assay

Since there was only a limited amount of native triple-helical collagen for addition to the collagenase assay solution under the optimum conditions of enzyme activity, it was impossible to detect the lower concentrations of substrate and productions in the solution with UV detection. Recently, a commercially available fluorogenic dye, NanoOrange, has been developed for quantitative detection of protein, employing dynamically non-covalent labeling of protein [22,23], and dynamic labeling CGE-LIF detection for proteins has been demonstrated using a running buffer containing NanoOrange [22]. As NanoOrange is a fluorescein-like dye, its non-covalent binding to the protein–SDS complex surface or hydrophobic domain of non-denatured proteins enhances the fluorescence sensitivity of protein due to conformational changes induced by binding. NanoOrange did not notably affect the protein–SDS complex mobility throughout the CGE separation, and its excitation wavelength (approximately 470 nm) was well suited for LIF detection with argon laser excitation (488 nm). As NanoOrange was anticipated to be compatible with the highly sensitive measurement of collagenase activity including the assessment of its inhibitors, we investigated the sensitive LIF detection for collagenase assay using NanoOrange.

As a higher concentration of SDS in the sieving matrix would increase the background fluorescence intensity and interfere with the binding between NanoOrange and protein, the SDS concentration had to be modified in order to obtain the desirable sensitivity for dynamic labeling CGE-LIF detection. Prior to investigating the separation condition, the effect of SDS concentration on fluorescence intensity for dynamic labeling was investigated using type II collagen. The SDS solutions, with final concentrations of 0, 0.05, 0.15 or 0.5%, were added to 1× NanoOrange solution containing or not containing type II collagen. Fluorescence intensities of blank solution (without collagen) and collagen sample solution (with collagen) for each concentration of SDS were determined using a spectrofluorometer. As shown in Fig. 1, the intensity of the blank solution increased with increasing SDS concentration. The net intensity of collagen fluorescence (△) was calculated by subtracting the blank fluorescence intensity for each SDS concentration. As a result, the net intensity of collagen fluorescence (△) was dramatically decreased above 0.15% SDS and the maximum difference was observed at 0.05% SDS. The concentration of SDS in the sieving matrix was set at 0.05% in order to maintain a sufficiently negative charge of proteins and prevent absorption of proteins on the capillary wall and polyacrylamide gel in the sieving matrix, although the fluorescence intensity would be higher at a lower concentration of SDS. It should be mentioned that this modification of SDS concentration is effective for increasing the sensitivity of dynamic labeling CGE-LIF detection using NanoOrange.

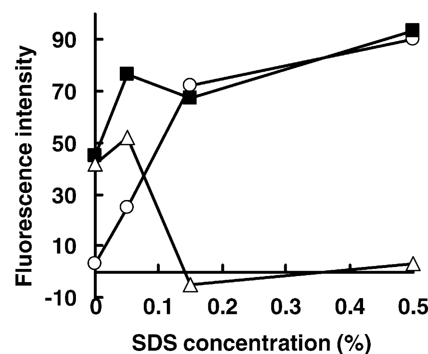

Fig. 1. Effect of SDS concentration (%) on fluorescence intensity of blank and type II collagen sample solution, and the net intensity of collagen fluorescence. *Keys*: blank solution (○); collagen sample solution (■); the net intensity of collagen fluorescence (△).

Next, the amount of NanoOrange in gel buffer was investigated over the range from 1× to 4× concentrations. Although the lower mass products (1/4 and 3/4 fragments) generated from collagenase could be detected using 1× concentration, the baseline of electropherograms could not be stablized in the CGE separation with LIF. When the amount of NanoOrange in gel buffer was set at 3× concentration, sufficient sensitivity for the products and a stable baseline were achieved. Also, the repeatability of peak areas of products was best using the gel buffer containing 3× concentration of NanoOrange as a running buffer.

3.2. CGE separation

CGE separation is affected by the composition of the sieving matrix, such as the kind of gel and its concentration. Among the gels for CGE separation, polyacrylamide is commonly used to prepare the sieving matrix, and the sieving matrix can be easily purchased and prepared as a gel buffer; however, the commercially available gel buffer could not be used in this study. Therefore, the composition of gel buffer was studied in order to completely separate and sensitively detect collagen and its products. As the result, the peaks of the collagen and its products, 1/4 and 3/4 fragments, in the enzyme reaction mixture could be separated and detected within 15 min using 50 mM AMPD–CACO buffer (pH 8.8) containing 4% polyacrylamide, 0.05% SDS and NanoOrange (3×) as a gel buffer (Fig. 2). The molecular weights of peaks at migration times of approximately 8.5 and 12.5 min, produced from MMP-13 reaction (electropherogram B) were calculated by plotting the log of the molecular weight against the reciprocal of the migration time using Mass markers Protein Test Mix under the same CGE conditions [24]. The molecular weights of the peak at 8.5 and 12.5 min of migration times were approximately 75 and 225 kDa, respectively. This revealed that the two peaks were the 1/4 fragment and 3/4 fragment of collagen, respectively. From the peak response in the electropherogram, it was better to estimate the enzyme activity using the peak area of the 3/4 fragment as an activity indicator, although both peaks of the fragments would be available for estimation of the activity. Also, the peak of MMP-13 was not detected under these separation conditions, because the concentration of MMP-13 was much lower than that of collagen in the reaction mixture.

To test the repeatability of the polyacrylamide gel buffer preparation method, four gel buffers were prepared in separate experiments and the peak areas and migration times of collagen and 3/4 fragment in reaction mixture were measured using 30 ng/tube MMP-13 and type II collagen. As the result, the relative standard deviations (R.S.D.) of the peak areas and the migration times of collagen and the 3/4 fragment were calculated to be less than 4.9 and 2.5%, respectively. The results indicated that the gel buffer preparation was reproducible. The modified dynamic labeling CGE-LIF method by NanoOrange was also reproducible.

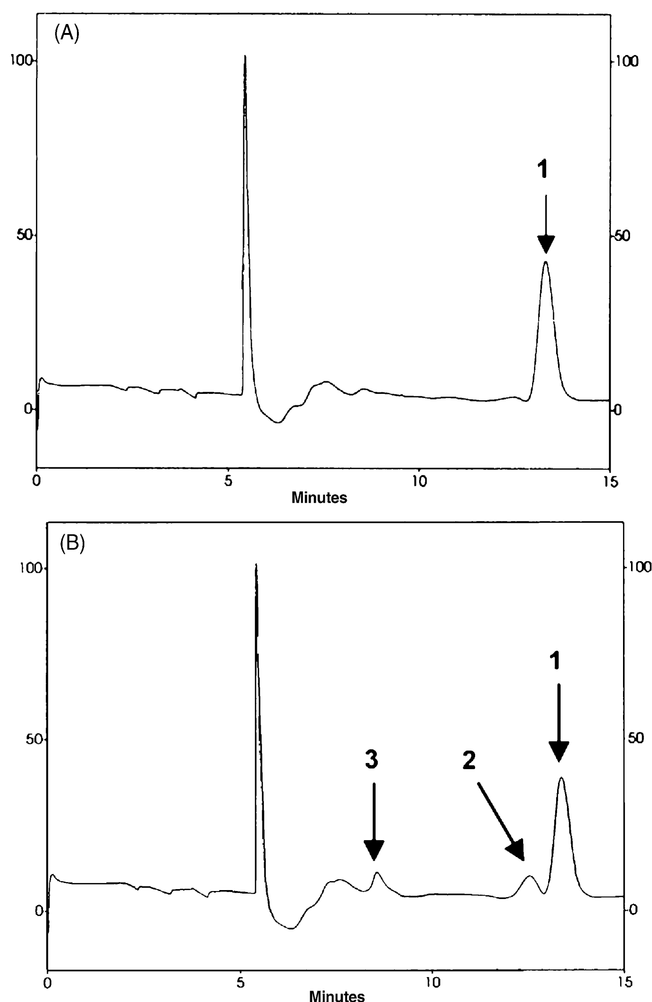

Fig. 2. CGE separation of type II collagen of the substrate and 1/4 and 3/4 fragments of the products in enzyme reaction solution without MMP-13 (A) and with 15 ng/tube MMP-13 (B). Capillary, coated capillary (100 μm i.d., effective length 20 cm); running gel buffer 4% polyacrylamide in 50 mM AMPD–CACO buffer (pH 8.8) containing 0.05% SDS and NanoOrange (3×); voltage, −8.1 kV; injection, 30 s at 0.5 psi; detection, excitation 488 nm, emission 590 nm. Peaks: (1) type II collagen; (2) 1/4 fragment; (3) 3/4 fragment.

3.3. Collagenase assays (MMP-13 assay)

The reaction conditions for the MMP-13 assay were investigated according a previously described method [5]. Fig. 3 shows the correlation between the incubation time of MMP-13 and the peak area of the 3/4 fragment using 15 ng/tube of MMP-13 at 37 °C. As the peak area of the 3/4 fragment increased linearly with an increase in the incubation time, a good correlation between the incubation time and the peak area of the 3/4 fragment was obtained up to the incubation

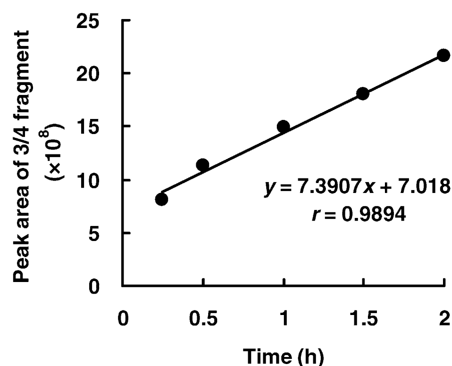

Fig. 3. Correlation between incubation time of collagenase cleavage of type II collagen and the peak area of the 3/4 fragment. MMP-13 reaction mixture was incubated at 37 °C using 15 ng/tube of MMP-13.

time of 2 h. As a result, the incubation time for the MMP-13 assay was set at 1 h.

Next, the correlation between the amount of MMP-13 and the peak area of the 3/4 fragment was investigated. In the result, a good linearity of the peak area of the 3/4 fragment was obtained over the range from 1.5 to 30 ng/tube of MMP-13 ($y \times 10^{-7} = 0.8064x - 2.7725$, $r = 0.9945$).

To test the repeatability of the MMP-13 assay, the peak areas of the fragment using 6 and 15 ng/tube of MMP-13 were measured repeatedly. R.S.D. of peak area were calculated to be less than 13.4% ($n = 10$ per amount level). As a result, the MMP-13 activity for evaluation of inhibitors by this method can be determined using 15 ng/tube, showing that the sensitivity of the assay would be enough high to be sufficient for HTS.

3.4. Collagenase assays (MMP-1 and MMP-8 assays)

Fig. 4 shows the typical electropherograms obtained from MMP-1 and MMP-8 enzyme reactions under the same separation conditions. The peaks of collagen and the 3/4 fragment could be identified by their migration times. In the collagenase assays, it was possible to estimate the enzyme activities using the peak areas of 3/4 fragments as activity indicators although the peaks of some impurities in type I collagen solution such as procollagen would be detected.

As the incubation time of MMP-1 and MMP-8 cleavage activity against type I collagen was optimized at 37 °C, the time was set at 2 h for MMP-1 and 1 h for MMP-8, respectively. Also, the correlation between the amount of collagenase and the peak area of the 3/4 fragment was investigated. In the result, a good linearity of the peak area of the 3/4 fragment was obtained over the range from 15 to 150 ng/tube of MMP-1 ($y \times 10^{-7} = 0.2006x + 4.7618$, $r = 0.9872$). Similarly, a good linearity of the peak area of the 3/4 fragment was obtained over the range from 3 to 30 ng/tube of MMP-8 ($y \times 10^{-7} = 0.944x - 1.2266$, $r = 0.9759$). These collagenase activities for evaluation of inhibitors can be determined using 90 ng/tube for MMP-1 and 30 ng/tube for MMP-8, respectively.

3.5. Evaluation of inhibitors

In order to use dimethylsulfoxide (DMSO) as an organic solvent for dissolving inhibitors, the effect of adding 2% DMSO on the assay solution was estimated prior to the evaluation of inhibitors according to a previously described procedure [21]. The results confirmed that addition of 2% DMSO did not affect these assays of enzyme activity and the CGE-LIF separation was not affected by the addition of DMSO. The IC_{50} values of the known MMPs inhibitors were determined by the established collagenase assays using 90 ng/tube of MMP-1, 30 ng/tube of MMP-8 or 15 ng/tube of MMP-13. As shown in Table 1, the IC_{50} values of the three inhibitors were close to those obtained by other assays

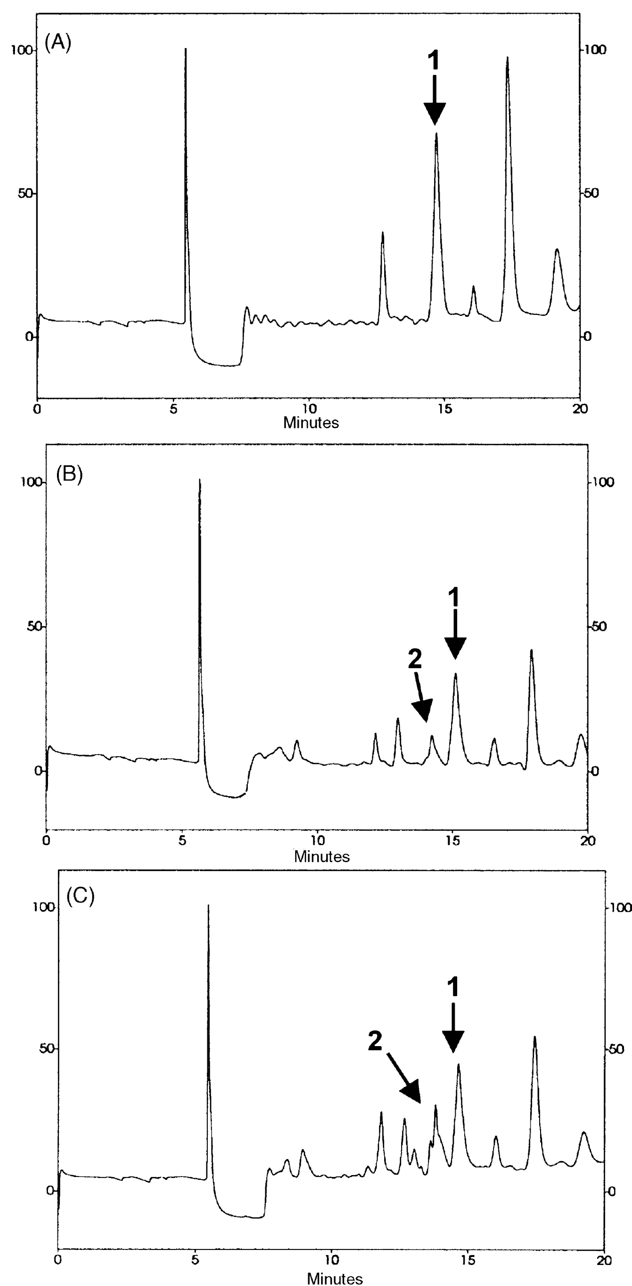

Fig. 4. CGE separation of type I collagen of the substrate and 3/4 fragment of the products in enzyme reaction solution without enzyme (A) and with 60 ng/tube MMP-1 (B) and 30 ng/tube MMP-8 (C). Capillary, coated capillary (100 μm i.d., effective length 20 cm); running gel buffer 4% polyacrylamide in 50 mM AMPD–CACO buffer (pH 8.8) containing 0.05% SDS and NanoOrange (3×); voltage, −8.1 kV; injection, 30 s at 0.5 psi; detection, excitation 488 nm, emission 590 nm. Peaks: (1) type I collagen; (2) 3/4 fragment.

[1,5] except for the values of METISAM against MMP-13 and MMI-166 against MMP-8 and MMP-13. We assumed that these IC_{50} values would differ between the two assays because of the usage of substrates having different characteristics (native substrate or the labeled peptide substrate) for the estimation. However, it is significant that the IC_{50} values of MMPs inhibitors against type I and II collagen

Table 1
Comparison of the IC_{50} values of inhibitors determined by CGE-LIF and other assays

Substrate	Enzyme	Compound	IC_{50} (nM)	
			CGE-LIF	Other assays
Type I collagen	MMP-1	MMI-166	>1000	>1000[a]
		METISAM	3320	3190[a]
		BB-2516	3.92	5[b]
	MMP-8	MMI-166	17.6	400[a]
		METISAM	9.42	7[a]
		BB-2516	1.96	2[b]
Type II collagen	MMP-13	MMI-166	5.46	0.8[a]
		METISAM	75.9	8[a]
		BB-2516	5.46	37[a]

[a] IC_{50} values were determined by the method of [5].
[b] IC_{50} values were obtained from [3].

could be estimated using the native triple-helical collagen substrate.

4. Conclusion

Dynamic labeling CGE-LIF methods have been developed for the determination of collagenase activity against type I and II collagen using native triple-helical collagen, without particular reagents such as antibodies and labeled substrates. The collagenase activities could be readily and sensitively estimated by measuring the 3/4 fragment of collagen in enzyme reaction mixtures. Also, the IC_{50} values of MMPs inhibitors could be precisely estimated under the preferable reaction conditions for collagenase. We expect that this method could be applied to crude samples such as cell extracts and tissue extracts, using sufficient amounts of collagen substrate to remain nearly unaffected by other various components, although an assay of crude samples was not attempted in this study.

The assay system described above can measure 50 samples a day and can be semi-automatically operated to examine more than 19,000 samples if this assay system is applied to a 384 capillary array system. The CE-LIF with a capillary array system should be very useful for developing HTS for drug discovery in the future.

References

[1] R.P. Beckett, A.H. Davidson, A.H. Drummond, P. Huxley, M. Whittaker, Drug Discov. Today 1 (1996) 16.
[2] T.M. Doherty, K. Asotra, D. Pei, H. Uzui, D.J. Wwilkin, P.K. Shah, T.B. Rajavashisth, Expert Opin. Ther. Patents 12 (2002) 665.
[3] M. Whittaker, C.D. Floyd, P. Brown, A.J.H. Gearing, Chem. Rev. 99 (1999) 2735.
[4] P.G. Mitchell, H.A. Magna, L.M. Reeves, L.L. Lopresti-Morrow, S.A. Yocum, P.J. Rosner, K.F. Geophegan, J.E. Hambor, J. Clin. Invest. 97 (1996) 761.
[5] C.G. Knight, F. Willenbrock, G. Murphy, FEBS 296 (1992) 263.
[6] G.N. Smith Jr., E.A. Mickler, K.A. Hasty, K.D. Brandt, Arthritis Rheum. 42 (1999) 1140.
[7] S. Hattori, H. Fujisaki, T. Kiriyama, T. Yokoyama, S. Irie, Anal. Biochem. 301 (2002) 27.
[8] S. Horstmann, P. Kalb, J. Koziol, H. Gardner, S. Wagner, Stroke 34 (2003) 2165.
[9] J.M. Milner, A.D. Rowan, S.-F. Elliott, T.E. Cawston, Arthritis Rheum. 48 (2003) 1057.
[10] K. Terato, Y. Nagai, K. Kawanishi, S. Yamamoto, Biochim. Biophys. Acta 445 (1976) 753.
[11] K. Sugiyama, K. Yamamoto, O. Kamata, N. Katsuda, Kurume Med. J. 27 (1980) 63.
[12] H. Yamada, K. Watanabe, T. Saito, H. Hayashi, Y. Niitani, T. Kikuchi, A. Ito, K. Fujikawa, L.S. Lohmander, J. Rheumatol. 26 (1999) 654.
[13] H.J. Isaaq, Electrophoresis 21 (2000) 1921.
[14] K. Govindaraju, D.K. Lloyd, J. Chromatogr. B 745 (2000) 127.
[15] X. Páez, L. Hernández, Biopharm. Drug Dispos. 22 (2001) 273.
[16] E. Carrilho, Electrophoresis 21 (2000) 55.
[17] L. Ma, X. Gong, E.S. Yeung, Anal. Chem. 72 (2000) 3383.
[18] L. Bousse, S. Mouradian, A. Minalla, H. Yee, K. Williams, R. Dubrow, Anal. Chem. 73 (2001) 1207.
[19] J. Wang, Electrophoresis 23 (2002) 713.
[20] D. Figeys, D. Pinto, Anal. Chem. 72 (2000) 330A.
[21] M. Sano, K. Ueno, H. Kamimori, J. Chromatogr. B 794 (2003) 149.
[22] L.J. Jin, B.C. Giordano, J.P. Landers, Anal. Chem. 73 (2001) 4994.
[23] M.D. Harvey, V. Bablekis, P.R. Banks, C.D. Skinner, J. Chromatogr. B 754 (2001) 345.
[24] K. Tsuji, J. Chromatogr. B 662 (1994) 291.