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Enzyme assay for protein kinase using micellar electrokinetic chromatography with laser-induced fluorescence detection

Masatake Sano*, Kyoji Ueno, Hiroshi Kamimori

Shionogi Research Laboratories, Shionogi & Co., Ltd., 12-4, Sagisu-5-Chome, Fukushima-ku, Osaka 553-0002, Japan

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Abstract

Micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) detection has been developed for a protein kinase assay. This protein kinase assay could readily determine the phosphorylation activity of substrate peptide kemptide using cAMP-dependent protein kinase (PKA) as a model enzyme. Kemptide and phosphorylated kemptide could be reacted with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorescence derivatization reagent for LIF detection by directly adding NBD-F into the PKA enzymatic reaction mixture. These derivatives of substrate and product were separated and detected within the analysis time of 5 min by micellar electrokinetic mode using a mixture of sodium dodecylsulfate and methanol as a running buffer. Good linearity of the peak response of the phosphorylated kemptide was obtained over the range of 1–20 mU/tube of PKA in the assay. The relative standard deviation of the peak areas of the phosphorylated kemptide using 2, 5 and 10 mU/tube of PKA were calculated to $<$10.4%, indicating that the assay was reproducible. Also, IC_{50} values of six PKA inhibitors, the K_i value and the inhibition pattern of one inhibitor, which were calculated to estimate by the variation of the peak area of the phosphorylated kemptide using 5 mU/tube of PKA, were consistent with the published data. The sensitivity of the assay was higher than that of enzyme-linked immunosorbent assay (ELISA) for PKA phosphorylation activity, as IC_{50} values, K_i value, and the inhibition mechanism of inhibitors could be estimated using one-tenth amounts of PKA, compared with that of ELISA. The MEKC–LIF is expected to be very useful for protein kinase assay and its application to the estimation of inhibitors because this method does not entail experimentally troublesome procedures such as the preparation of antibody or fluorescence-labeled substrate.

Keywords: Enzymes; Protein kinase

1. Introduction

Protein kinases play a significant role in intracellar signal transduction processes. Various protein kinase assays for evaluating the activity of inhibitors have been developed for drug discovery in many pharmaceutical companies. To estimate the inhibition activity of more than 100 000 new candidate drugs rapidly and automatically, high-throughput screening (HTS) has been developed.

Scintillation proximity assay (SPA) has been widely applied to HTS in many industries and the rapid assay of protein kinase activity by SPA with (γ-^{32}P) ATP has been reported [1,2]. However, there are risks of contamination and radiation exposure when using radioactive reagents. To overcome these disadvantages, many nonradioisotopic immunoassays

*Corresponding author. Fax: +81-6-6458-0987.
E-mail address: masatake.sano@shionogi.co.jp (M. Sano).

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using chemiluminescent detection or homogeneous time-resolved fluorescence detection have been recently developed for HTS [3–6]. Although multi-samples can be rapidly estimated using microtiter plates with a robotic system in these non-radioisotopic assays, antibody must be prepared or obtained for the assay. Since the establishment of protein kinase assay would entail somewhat troublesome procedures, it would be important to develop more convenient assays for discovering drug candidates.

Capillary electrophoresis (CE) is an excellent and convenient analytical tool offering the advantage of high separation efficiency, as previously reported elsewhere [7–11], and it has been widely applied to determine enzyme activity [11–16]. Additionally the array system based on CE is applicable to multiple assays, such as DNA sequencing [17], and Yeung et al. have already developed an enzyme assay using the CE array system [16]. Thus, the CE technique should be useful for protein kinase assay in drug discovery, as it could be applied to HTS.

The objective of our study was to establish a CE method with laser-induced fluorescence (LIF) detection for protein kinase assays to evaluate the activity of inhibitors for drug discovery. As the sensitivity of LIF detection is thought be higher than that of ultraviolet detection, a highly sensitive enzyme assay should be useful for the CE–LIF method, leading to a reduction in the amount of enzyme needed for the assay.

In this work, we developed a method using CE–LIF for the protein kinase assay using cAMP-depended protein kinase (PKA) as a model enzyme. Our method involves determining the phosphorylation activity of kemptide of the substrate reacting with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorescence derivatization reagent by directly adding NBD-F into an PKA enzymatic reaction mixture. These derivatives of the substrate and the product can be separated and detected within an analysis time of 5 min by micellar electrokinetic capillary chromatography (MEKC) using a mixture of sodium dodecylsulfate and methanol as a running buffer. The sensitivity of the assay was higher than that of enzyme-linked immunosorbent assay (ELISA) [3] for PKA phosphorylation activity, and IC_{50} values, K_i value, and the inhibition pattern of its inhibitors could be estimated.

2. Experimental

2.1. Reagents

Adenosine 5′-triphosphate (ATP) disodium salt, adenosine 3′,5′-cyclic monophosphate sodium salt (cAMP), cAMP dependent protein kinase (PKA), Kemptide (Kem) acetate salt, polyoxyethylene 23 lauryl ether (Brij 35), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) dihydrochloride, *N*-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfoamide (H-89) dihydrochloride, 1-(5-isoquinolynylsulfonyl)-piperazine (HA-100) and *N*-(2-guanidinoethyl)-5-isoquinoline-sulfonamide (HA-1004) were purchased from Sigma (St. Louis, MO, USA). *N*-(2-Aminoethyl)-5-chloronaphthalene-1-sulfonamide (A-3) hydrochloride and KT-5720 were purchased from Biomol Research Labs. (Plymouth Meeting, PA, USA). 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) were purchased from Dojindo Labs. (Kumamoto, Japan). Magnesium acetate tetrahydrate and dimethylsulfoxide (DMSO) was obtained from Nacalai Tesque (Kyoto, Japan). Sodium dodecylsulfate (SDS), 2 *M* sodium hydroxide and boric acid were purchased from Wako (Osaka, Japan). Sodium tetraborate decahydrate was purchased form Cica (Tokyo, Japan). Methanol, acetonitrile and water were of HPLC grade. All other chemicals were of reagent grade.

2.2. Apparatus

A P/ACE 5510 CE system equipped with an argon ion laser emitting 488 nm and an LIF detector (emission wavelength filter of 530 nm) (Beckman Instruments, Fullerton, CA, USA) was used. An untreated fused-silica capillary (Supelco) with 75 μm I.D. 360 μm O.D. of 27 cm (20 cm to detector window) was assembled in the LIF cartridge format, which was applied constant voltage (15 kV) for 5 min at 20 °C for the MEKC separation. The running buffer used was 20 m*M* sodium borate buffer (pH

9.0) containing 20 m*M* SDS and methanol (40:7, v/v). Samples were injected by pressure for 5 s at 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa) Prior to injection, the capillary was washed with running buffer for 30 s at each run. The electropherographic data were analyzed with Beckman P/ACE STATION software (version 1.0).

A Shimadzu LC-2010C HPLC system (Kyoto, Japan) was used to purify the phospholylated Kemptide. Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) used was a Bruker Daltonics Reflex III (Bremen, Germany).

2.3. Preparation of phosphorylated kemptide

Phosphorylated kemptide (Kem-P) was prepared according to the method of Meyer et al. [18]. PKA 1000 units/ml in 0.6 g/dl dithiothreitol (50 μl) was incubated with 20 μl of 50 μ*M* cAMP for 10 min at 30 °C. The PKA mixture was added to 260 μl of 50 m*M* HEPES buffer (pH 7.5) containing 25 m*M* magnesium acetate and 20 μl ATP (10 mg/ml) in water, and 50 μl of Kem (1.25 m*M*) in 0.1 g/dl Brij 35. The mixture was incubated for 30 min at 30 °C, and then 2% TFA (400 μl) was added to the PKA reaction mixture. A 100-μl aliquot of the mixture was injected onto the HPLC system with a YMC Pack ODS-AQ (150×3.0 mm, 5 μm) (YMC, Kyoto, Japan) several times. The ultraviolet wavelength of the detector was set at 220 nm. The mobile phases used were 0.1% trifluoroacetic acid solution (Solvent A) and acetonitrile containing 0.1% trifluoroacetic acid/water (84:16, v/v) (Solvent B), which were delivered at a total flow-rate of 0.4 ml/min. The mobile phase gradient program was set as follows. After injection, 95% of Solvent A was initially maintained over 4 min. Next, a linear gradient was used starting with 95% of Solvent A over 20 min until Solvent A–Solvent B (80:20) was attained, after which a successive linear gradient was employed consisting of Solvent A–Solvent B (80:20) to 100% of Solvent B up to 25 min. The peak corresponding to Kem-P was eluted around the retention time of 18 min and was completely separated from that of Kem. The peak fraction corresponding to Kem-P was lyophilized, and the product obtained from the fraction was identified with MALDI-TOF MS. As a result, the analysis by MALDI-TOF MS of the product revealed the presence of the molecular ion at m/z 852.5 $[M+H]^+$, corresponding to the molecular mass of Kem-P.

2.4. PKA assay

To 5 μl of PKA solution (1–20 mU, in 50 m*M* HEPES buffer (pH 7.5) containing 0.02 g/dl Brij 35 and 25 m*M* magnesium acetate) was added 5 μl of 5 μ*M* cAMP in distilled water followed by preincubation for 5 min at 30 °C. Next, 10 μl of 31 μ*M* Kem (in 50 m*M* HEPES buffer (pH 7.5) containing 0.01 g d/l Brij 35 and 25 m*M* magnesium acetate) containing 9 μ*M* ATP was added to the mixture, which was incubated for 2 h at 30 °C. To the mixture, 5 μl of 50 m*M* NBD-F in acetonitrile was added followed by heating for 3 min at 30 °C. To the reaction mixture was added 80 μl of 20 m*M* sodium borate buffer (pH 9.0), and the solution was subjected to the MEKC–LIF system.

The peak areas of the NBD-derived phosphorylated Kem (NBD–Kem-P) were monitored as an indicator of PKA activity.

2.5. Effect of DMSO on PKA activity

To 5 μl of PKA solution (5 mU, in 50 m*M* HEPES buffer (pH 7.5) containing 0.02 g/dl Brij 35 and 25 m*M* magnesium acetate) was added 5 μl of 5 μ*M* cAMP in distilled water, followed by preincubation for 5 min at 30 °C. Next, 0.1–1 μl of DMSO and 10 μl of 31 μ*M* Kemptide (in 50 m*M* HEPES buffer (pH 7.5) containing 0.01 g d/l Brij 35 and 25 m*M* magnesium acetate) containing 9 μ*M* ATP were added to the mixture. The final percentages of volume of the added DMSO into PKA assay solution (20 μl) were approximately 0.5–5%. After the mixture was incubated for 2 h at 30 °C, derivatization with NBD-F and MEKC–LIF analysis were carried out as described above.

2.6. Determination of IC_{50} value and K_i value

To 5 μl of PKA solution (5 mU, in 50 m*M* HEPES buffer (pH 7.5) containing 0.02 g/dl Brij 35

and 25 m*M* magnesium acetate) was added 5 μl of 5 μ*M* cAMP in distilled water, followed by preincubation for 5 min at 30 °C. A 1-μl volume of inhibitor in DMSO and 10 μl of 31 μ*M* Kemptide (in 50 m*M* HEPES buffer (pH 7.5) containing 0.01 g d/l Brij 35 and 25 m*M* magnesium acetate) containing 9 μ*M* ATP were added to the mixture. After the mixture was incubated for 2 h at 30 °C, derivatization with NBD-F and MEKC–LIF analysis were carried out as described above. IC_{50} values were determined by PKA activity analyses in the presence of several concentrations of each inhibitor. Also, the K_i value of HA-1004 was calculated from the results of the Lineweaver–Burk plot.

3. Results and discussion

3.1. Optimization of NBD derivatization of Kem-P

In order to sensitively detect Kem-P in a PKA reaction mixture with fluorescence detection by LIF, NBD-F was chosen as the fluorescence derivative reagent. NBD-F is widely used as an amino-reactive fluorescence reagent and can be rapidly reacted with amino groups under mild conditions [19–22]. Furthermore, the excitation wavelength of the NBD derivatives is approximately 470 nm, which is suited for that of the argon ion laser (488 nm).

To optimize the derivatization of Kem-P with NBD-F, the effect of pH of the PKA reaction mixture on derivatization was examined using the Kem-P. While the optimum pH of NBD derivatization for peptide and amino acid is 8 [20,21], the peak area of the NBD–Kem-P was constant at pH between 7 and 9 in the reaction mixture. As a result, the NBD derivatization of Kem-P could be performed by direct addition of NBD-F to the PKA reaction mixture, because the pH of the PKA reaction mixture was approximately 7.5. As the reaction condition was additionally optimized for temperature, reaction time and the concentration of NBD-F using the Kem-P, the peak area of NBD–Kem-P reached a plateau when the Kem-P was reacted with 5 μl of 50 m*M* NBD-F in acetonitrile at 30 °C for 3 min. This fluorescence derivatization was very useful for rapid assay because the derivatization reaction was instantly completed in the mixture under mild conditions immediately after PKA enzyme reaction and the enzyme reaction could be terminated by the addition of NBD-F into the mixture.

Using the conditions established above, the correlation between the concentration of Kem-P (x) and the peak area of NBD–Kem-P (y) gave good linearity over the range 0.03–3 μ*M* of Kem-P ($y = 6830332x - 13081$, $r = 0.9998$). It also confirmed that the peak area of NBD–Kem-P derived from the Kem-P was almost equal to that of the direct addition of NBD-F to PKA reaction mixture over the assay range, when the equivalent amount of Kem was used.

3.2. CE separation

As a beginning, capillary zone electrophoresis was examined using an uncoated fused-silica capillary with an alkalinity buffer as a running buffer that could bring about electroosmotic flow. However, the peak of the NBD derivative of Kem (NBD–Kem) was not detected because the derivative would be absorbed onto the capillary wall. To prevent the absorption, MEKC mode was studied using a running buffer containing SDS. While the absorption of NBD–Kem onto the capillary wall hardly occurred and the peak of NBD–Kem could be detected, the peaks of the NBD–Kem and the NBD–Kem-P could not be sufficiently separated. Next, the effect in addition of organic solvent to the running buffer was investigated in order to separate the two peaks over a short analysis time. As shown in Fig. 1, the peaks of two derivatives in PKA reaction mixture could be separated and detected within 5 min using 20 m*M* sodium borate buffer (pH 9.0) containing 20 m*M* SDS–methanol (40:7, v/v) as a running buffer.

3.3. PKA assay

3.3.1. Incubation time

Fig. 2 shows the correlation between the incubation time of phosphorylation of Kem and the peak area of NBD–Kem-P using 5 and 10 mU/tube of PKA. As the peak area of NBD–Kem-P increased linearly with incubation time, a good correlation between the incubation time and the peak area of NBD–Kem-P was obtained up to the incubation time

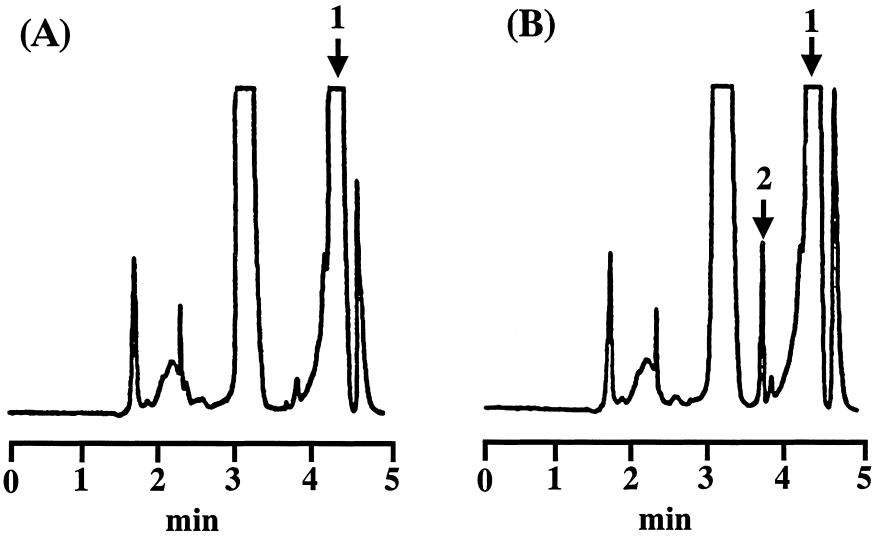

Fig. 1. MEKC separation of Kem of the substrate and Kem-P of the product in enzyme reaction mixture using 31 μ*M* Kem without PKA (A) and with 5 mU PKA (B). Capillary, fused-silica capillary (75 μm I.D., 20/27 cm); running buffer, 20 m*M* sodium borate buffer (pH 9.0) containing 20 m*M* SDS–methanol (40:7, v/v); voltage, 15 kV; injection, 5 s at 0.5 p.s.i.; detection, Ex: 488 nm, Em: 530 nm. Peaks: 1 = NBD–Kem; 2 = NBD–Kem-P.

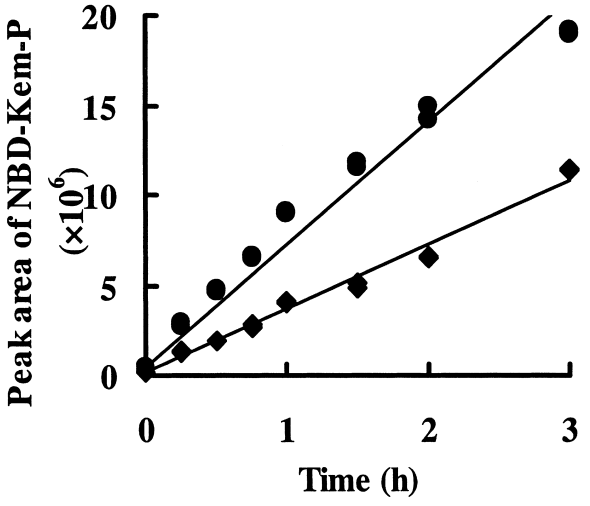

Fig. 2. Correlation between the incubation time of phosphorylation of Kem and the peak area of NBD–Kem-P. Keys; 5 mU/tube of PKA (♦), 10 mU/tube of PKA (●). PKA reaction mixture was incubated at 30 °C followed by the reaction with NBD-F for 3 min at 30 °C.

of 3 h. As a result, the incubation time for PKA assay was set at 2 h.

3.3.2. Amount of PKA

As Fig. 3 shows the correlation between the amount of PKA and the peak area of NBD–Kem-P, good linearity of the peak area of the NBD–Kem-P was obtained over the range from 1 to 20 mU/tube of PKA. The PKA activity for evaluation of inhibitors in this method can be determined using 5 mU/tube of PKA, showing that the sensitivity of the assay is tenfold higher than that of ELISA [3].

3.3.3. Precision

To test the reproducibility of the PKA assay, the peak areas of the NBD–Kem-P using 2, 5 and 10 mU/tube of PKA were measured repeatedly. Within-run precision was evaluated by assay of replicates ($n = 10$ per amount level and run) over three runs, and between-run precision by all of the results of the three runs ($n = 30$ per amount level). As shown in Table 1, the within-run and between-run precisions were $< 10.4\%$ and $< 10.8\%$, respectively, indicating that this method was reproducible for the PKA assay.

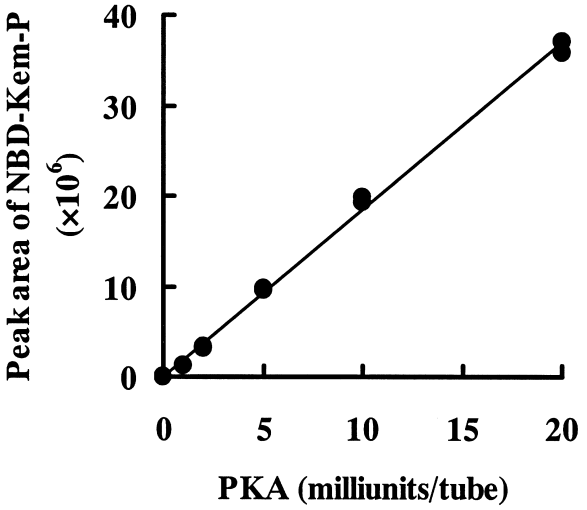

Fig. 3. Correlation between the amount of PKA and the peak area of NBD–Kem-P. PKA reaction mixture was incubated at 30 °C followed by the reaction with NBD-F for 3 min at 30 °C.

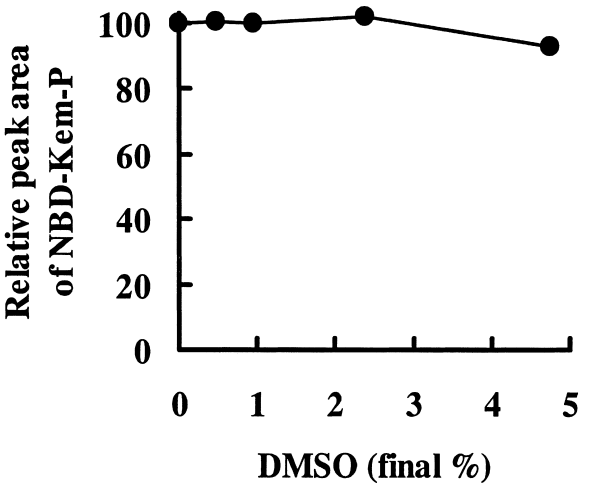

Fig. 4. Effect of DMSO concentration of PKA reaction mixture on the its activity. PKA reaction mixture was incubated at 30 °C followed by the reaction with NBD-F for 3 min at 30 °C.

3.4. Evaluation of inhibitors

3.4.1. Effect of DMSO on PKA activity

To assess inhibitors for drug discovery, DMSO is often used as an organic solvent for dissolving inhibitors. We estimated the effect of adding DMSO on the PKA activity prior to the evaluation of inhibitors. Fig. 4 shows the relationship between the relative peak area of NBD–Kem-P and the final percentage of the added DMSO into PKA assay solution (20 μl). The relative peak area of NBD–Kem-P was almost not decreased even though approximately 5% DMSO had been added into the assay solution, indicating that addition of DMSO did not affect PKA activity, and DMSO can be used as an organic solvent for dissolving inhibitors in this assay. It has also confirmed that the MEKC–LIF separation was not affected by the addition of DMSO.

Table 1
Precision of the PKA assay by MEKC–LIF

Run	*n*	Precision (RSD[a],%)		
		Amount of PKA (mU/tube)		
		2	5	10
1	10	6.1	8.1	10.4
2	10	2.9	5.6	3.9
3	10	4.3	8.2	4.2
Total	30	4.7	9.3	10.8

[a] Relative standard deviation.

3.4.2. Determination of IC_{50} value and K_i value

The IC_{50} values and K_i value of the known kinase inhibitors were determined by this PKA assay using 5 mU/tube of PKA. As shown in Table 2, IC_{50} values of six inhibitors were close to those in the literature.

Further, the determination of the K_i value and the evaluation of the inhibition mechanism of HA-1004 were carried out by Lineweaver–Burk plot. As

Table 2
Comparison of the IC_{50} values of inhibitors determined by MEKC–LIF with IC_{50} values and K_i values in the literature

Compound	IC_{50} (μ*M*)		K_i (μ*M*)[b]
	MEKC–LIF	chemiluminescent assay[a]	
H-7	7	10	3
H-89	0.02	0.057	0.048
A-3	11	–[c]	4.3
HA-100	5	2	–
HA-1004	5	–	2.3
KT5720	0.9	1.3	0.056

[a] IC_{50} values were obtained from Ref [3].
[b] K_i values were obtained from Refs. [23–26].
[c] No literature data was found.

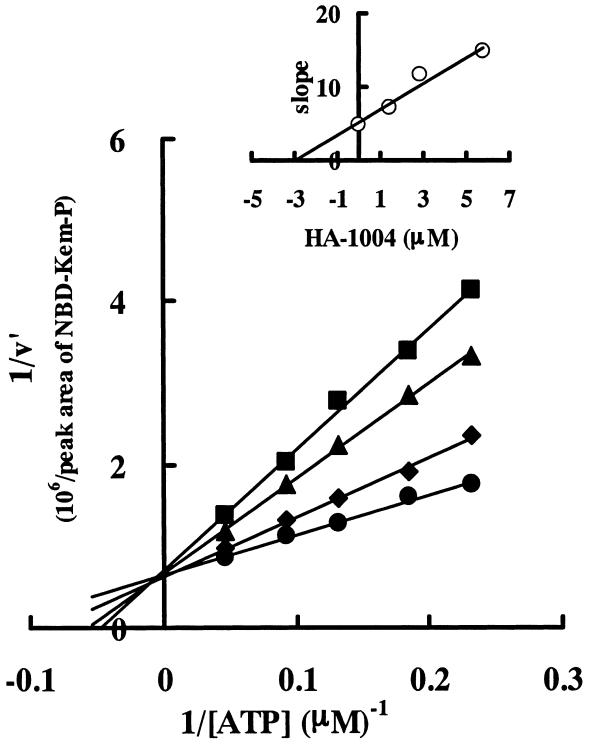

Fig. 5. Lineweaver–Burk plot in inhibition of PKA activity by HA-1004. PKA reaction mixture was incubated for 2 h at 30 °C using 5 mU/tube of PKA and derivatization of NBD was allowed for 3 min at 30 °C. The final concentrations of HA-1004 in the reaction mixture were 0 (●), 1.44 (♦), 2.89(▲) and 5.78 μ*M* (■). The concentration of ATP was 4.3, 5.4, 7.6, 10.8 and 21.6 μ*M*. Inset shows secondary plot of the slope of the line as a function of HA-1004 concentration.

shown in Fig. 5, the kinetic analysis revealed that the inhibition mechanism of PKA by HA-1004 was competitive with ATP, and the K_i value was 3 μ*M*. These results corresponded to the published data [23–26]. Thus, IC_{50} values, K_i value, and the inhibition mechanism of kinase inhibitors could be estimated using one-tenth the amount of PKA, compared with ELISA [3], indicating a much higher sensitivity of the assay in comparison with ELISA.

4. Conclusion

The MEKC–LIF method has been developed for the determination of PKA activity without antibodies, radioisotope and labeled substrates. PKA activity could be readily estimated by measuring the fluorescence derivative of phosphorylated Kemptide. Also IC_{50} values, K_i value and the inhibition mechanism of kinase inhibitors could be precisely estimated using only a small amount of PKA. The proposed MEKC–LIF method should aid the rapid establishment of a protein kinase assay

The assay system described above can measure 200 samples a day including MEKC analysis and sample preparation such as PKA reaction and NBD-derivatization, and it is not impossible to examine more than 19 000 samples if this assay system is applicable to 96 capillaries array system. The MEKC–LIF with capillary array system should be expected to be very useful for developing HTS for the discovery of inhibitors in the future.

References

[1] D.B. Evans, K.B. Rank, S.K. Sharma, J. Biochem. Biophys. Methods 50 (2002) 151.
[2] N.D. Cook, Drug Discov. Today 1 (1996) 287.
[3] C. Lehel, S.D. Issakani, M. Brasseur, B. Strulovici, Anal. Biochem. 244 (1997) 340.
[4] J. Mosier, C.E. Olesen, J.C. Voyta, I. Bronstein, Methods Enzymol. 305 (2000) 410.
[5] K. Enomoto, Y. Aono, T. Mitsugi, K. Takahashi, R. Suzuki, M. Preadat, G. Mathis, G. Kominami, H. Takemoto, J. Biomol. Screen. 5 (2000) 263.
[6] B. Bader, E. Butt, A. Palmetshofer, U. Walter, T. Jarchau, P. Drueckes, J. Biomol. Screen. 6 (2001) 255.
[7] H.J. Issaq, Electrophoresis 21 (2000) 1921.
[8] K. Govindaraju, D.K. Lloyd, J. Chromatogr. B 745 (2000) 127.
[9] X. Páez, L. Heránndez, Biopharm. Drug Dispos. 22 (2001) 273.
[10] D. Figeys, D. Pinto, Anal. Chem. 72 (2000) 330A.
[11] J. Wang, Electrophoresis 23 (2002) 713.
[12] J.F. Dawson, M.P. Boland, C.F.B. Holmes, Anal. Biochem. 220 (1994) 340.
[13] W.S. Wu, J.L. Tsai, Anal. Biochem. 269 (1998) 423.
[14] D.M. Snow, J.H. Shaper, N.L. Shaper, G.W. Hart, Anal. Biochem. 271 (1999) 36.
[15] S. Bhoopathy, M. Sarker, H.T. Karnes, J. Pharmaceut. Biomed. Anal. 25 (2001) 721.
[16] L. Ma, X. Gong, E.S. Yeung, Anal. Chem. 72 (2000) 3383.
[17] E. Carrilho, Electrophoresis 21 (2000) 55.
[18] H.E. Meyer, E.H. Posorske, H. Korte, L.M.G. Heilmeyer Jr., FEBS 204 (1986) 61.
[19] K. Imai, Y. Watanabe, Anal. Chem. Acta 130 (1981) 377.
[20] Y. Watanabe, K. Imai, Anal. Biochem. 116 (1981) 471.

[21] H. Kanazawa, T. Nagatsuka, M. Miyazaki, Y. Matsushima, J. Chromatogr. A 763 (1997) 23.
[22] I. Beijersten, D. Westerlund, J. Chromatogr. A 716 (1995) 389.
[23] H. Hidaka, M. Inagaki, S. Kawamoto, Y. Sasaki, Biochemistry 23 (1984) 5036.
[24] T. Chijiwa, A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, H. Hidaka, J. Biol. Chem. 265 (1990) 5267.
[25] M. Inagaki, S. Kawamoto, H. Itoh, M. Saitoh, M. Hagiwara, J. Takahashi, H. Hidaka, Mol. Pharmacol. 29 (1986) 577.
[26] H. Kase, K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato, M. Kaneko, Biochem. Biophys. Res. Commun. 30 (1987) 436.