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Fluorescence-Labeled Peptide p*I* Markers for Capillary Isoelectric Focusing

Kiyohito Shimura,*,‡ Kei-ichiro Kamiya,‡ Hiroyuki Matsumoto,†,§ and Ken-ichi Kasai‡

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan, and Laboratory of Molecular Biophotonics, 5000 Hirakuchi, Hamakita, Shizuoka 434-8555, Japan

Nineteen fluorescent pH standards or p*I* markers ranging pH 3.64−10.12 were developed for use in capillary isoelectric focusing using laser-induced fluorescence detection. Tetra- to tridecapeptides containing one cysteine residue were designed to focus sharply at their respective isoelectric points by including amino acids that contain charged side chains, the pK_a values of which are close to the corresponding p*I* values. An iodoacetylated derivative of tetramethylrhodamine was coupled to the thiol group of cysteine to yield fluorescent p*I* markers. The p*I* values of the labeled peptides were precisely determined after isoelectric focusing on polyacrylamide gel slabs by direct measurement of the pH of the focused bands. The markers were subjected to capillary isoelectric focusing for 10−15 min in coated capillaries under conditions of low electroosmosis and were detected by means of a scanning laser-induced fluorescence detector down to a level of subpicomolar range. The markers permitted the calibration of a wide-range pH gradient formed in a capillary by fluorescence detection for the first time and should facilitate the development of highly sensitive analytical methods based on a combination of capillary isoelectric focusing and laser-induced fluorescence detection.

Isoelectric focusing (IEF) is a high-resolution separation technique for use in the detection and identification of proteins.[1] Carrier ampholytes in the IEF separation medium automatically form a pH gradient in an electric field and the amphoteric analytes accumulate at the points where the pH of the medium is equal to their isoelectric point (p*I*). The capillary format of IEF (CIEF) has several distinct features that are not shared by the conventional IEF, which employs polyacrylamide or agarose gels. That is, separation is rapidly achieved in a free solution under high field strength starting from a homogeneous mixture of a sample and carrier ampholytes filled in a capillary, and the separated species can be quantitatively detected with an on-capillary detector. Because of the absorption of short-wavelength ultraviolet (UV) light by the currently available carrier ampholytes themselves, the sensitivity of detection for CIEF using an UV absorption detector is compromised. When CIEF is interfaced with laser-induced fluorescence (LIF) detection, the full scope of its ability as a sensitive, quantitative, and rapid separation technique becomes available.[2,3] Although most analytes must be derivatized to be fluorescent, capillary isoelectric focusing (CIEF) with LIF detection represents a new platform that has great potential for the development of novel analytical schemes for microscale analyses with both high resolution and high sensitivity. As an example, it has been demonstrated that the isoforms of a protein antigen at a picomolar level can be separated and detected as complexes with a fluorescence-labeled antibody fragment.[4] Other examples involve the quantification of protease activity at trace levels by detecting a product of peptidolytic action on a dye-labeled peptide substrate[5] and the simultaneous multiple analyte detection using fluorescent peptide indicators with different p*I*'s.[6]

The pH gradient, once formed in a capillary, is not completely stable and gradually decays as the result of anodic and cathodic drift due to the charge on carrier ampholytes to balance proton and hydroxyl ions in acidic and basic regions, respectively.[1,7] It is also influenced by electroendoosmosis of the capillary to varying degrees, depending on the nature of the inner surface of the capillary.[7−9] Thus, to use this separation technique at its maximum potential and identify fluorescent analytes by their p*I* values, it is very important to develop a basis for monitoring the shape of the pH gradient formed in the capillary. For this purpose, proteins that have established p*I* values have been used in slab gel IEF as standard substances, that is, p*I* markers. The number of naturally fluorescent proteins that can be excited with typically used visible lasers is limited, and the labeling of proteins at their amino groups with a fluorescent dye would likely produce heterogeneous

* To whom correspondence should be addressed. Fax: +81-426-85-3742. E-mail: shimurak@pharm.teikyo-u.ac.jp.

‡ Teikyo University.

§ Laboratory of Molecular Biophotonics.

† Present address: Central Research Laboratory, Hamamatsu Photonics K.K., 5000 Hirakuchi, Hamakita, Shizuoka 434-8601, Japan.

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products as a result of the variation of the number and the sites of the label. We have previously reported on the fluorescent-dye labeling of commercially available peptides at their N termini for use as fluorescent p*I* markers.[2] Although these labeled peptides enabled us to calibrate the pH gradient by fluorescence detection for the first time, the utility of these labeled peptides was limited by the narrow pH range covered and their poor solubility due to the presence of amino acid residues having hydrophobic side chains in the original peptides. The development of fluorescent pI markers was also reported by two separate groups, one of which used dansylated peptides and some dansylated components of commercially available carrier ampholytes.[10] The other used low-molecular-mass compounds that can be excited with UV light, although their chemical entities were not reported.[11] UV-induced fluorescence, however, is not suitable for highly sensitive detection as a result of autofluorescence of both biological samples and the commercially available carrier ampholytes themselves.

On the other hand, we found that specifically designed synthetic peptides, tri- to hexapeptides, each having one tryptophan residue and in which other amino acid residues contained an ionic side chain, can be used as a set of good p*I* markers for CIEF with UV-absorption detection over the pH range from 3.38 to 10.17 with a maximum p*I* gap of 1.13 pH units.[12,13] On the basis of the success of the peptide p*I* markers for UV-absorption detection, we initiated a project to develop a set of fluorescent p*I* markers by labeling synthetic peptides that were specifically designed for this purpose. For rapid and sharp focusing, a peptide should have an ionic group or groups, the acid dissociation constant of which is close to its p*I* value.[5,12] Preliminary trials showed that the presence of an N-terminal amino group in the free form is crucial for neutral to weakly basic p*I* markers to be focused well. The labeling of the N-terminal amino group was therefore abandoned, and the labeling of the thiol group of a cysteine residue was used instead. In some cases, the presence of a single free N-terminal amino group was not sufficient, and branched peptides with multiple N termini were found to provide even better focusing properties. We developed 19 fluorescent markers and determined their p*I* values precisely. These fluorescent markers covered a pH range of 3.64−10.12 with a maximum gap of 0.68 pH units.

Since the mobility of the focused ampholyte reaches a minimum after the focusing conditions are attained, the resulting pH gradient must be mobilized when detection is carried out with a single-point static detector.[7−9] The mobilization process represents an additional factor for irreproducibility and might negatively affect the quality of CIEF separation as a whole. To solve this problem, a complete capillary detection system for CIEF by scanning using UV absorption[14] or LIF[15] and by imaging using UV absorption[16] or LIF[17] has been developed. Although scanning requires a longer time than imaging to obtain a complete electropherogram, the necessary alterations required for a static detector can be small. The static detector that was used in previously reported analyses[2,4,5,18] was modified, and a scanning LIF detector for CIEF has been developed. In this procedure, all the electrophoresis components, including the separation capillary and electrodes, are mounted on an electric translational stage and are moved against stationary LIF detection optics. The developed fluorescent p*I* markers are focused sharply within 10 min and are detected by the scanner within 1 min at a detection limit of <1 pM, which was even lower than that of the LIF detection system used in a static mode.[2,5] These markers should facilitate the development of highly sensitive analytical techniques at a microscale in the CIEF-LIF platform.

EXPERIMENTAL SECTION

Chemicals and Materials. The following chemicals and materials were obtained from commercial sources: Synthetic peptides (Peptide Institute, Inc., Minoh, Osaka, Japan); Pharmalyte (Pharmacia Biotech AB, Uppsala, Sweden); tetramethylrhodamine 5-iodoacetamide (Molecular Probes, Eugene, OR, Catalog no. T-6006); methylcellulose (Sigma Chemical Company, St. Louis, MO); a reversed-phase chromatographic column (TSK-Gel ODS-80Ts, 4.6 mm i.d. × 25 cm) and a cartridge guard column (TSK guard gel ODS-80TS, 3.2 mm i.d. × 1.5 cm) (Tosoh Corp., Tokyo); fused-silica capillaries (GL Sciences Inc., Tokyo); μSIL-FC capillaries (J &W Scientific, Folsom, CA). Peracetic acid at about 1 M was prepared from acetic anhydride and hydrogen peroxide according to the published procedure.[19]

Purification of Dye. Tetramethylrhodamine 5-iodoacetamide from the supplier was further purified by reversed-phase chromatography. The reactive dye, 5 mg, was dissolved in 2 mL of acetonitrile/water (1:1 (v/v)), and the solution was centrifuged at 10000*g* for 5 min. A 100-μL aliquot of the supernatant was applied to a reversed-phase column (TSK-Gel ODS-80Ts, 4.6 mm i.d. × 25 cm, Tosoh Corp., Tokyo, Japan) with a cartridge guard column (TSK guardgel ODS-80Ts, 3.2 mm i.d. × 1.5 cm, Tosoh Corp., Tokyo, Japan) equilibrated with 25% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. An acetonitrile gradient of 25−55% over a 30-min period was applied just after the application of a sample. The eluate was monitored by absorption at 280 nm, and the main peak, which appeared at 22−24 min, was collected. The concentration of dye was determined by measurement of the absorbance at 549 nm in 20% methanol (v/v) based on an extinction coefficient of 87 000.[20] The purified dye was dried by evaporation in 25-nmol aliquots using a centrifugal concentrator.

Labeling of Peptides. The purified dye (25 nmol) was dissolved in 20 μL of acetonitrile and mixed with 80 μL of 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA. A 1-μL portion of a peptide solution (50 mM) was added to the dye solution and allowed to react at room temperature overnight in the dark. The reaction mixture was acidified by the addition of 15 μL of 1 M HCl and subjected to reversed-phase column chromatography under the same conditions as described for the purification of the dye. A fraction of the main peak accounting

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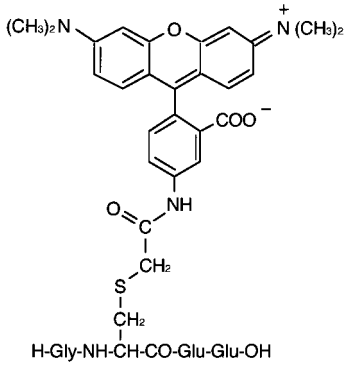

Figure 1. Structure of the labeled peptide no. 58.

for more than 80% of the total peak area was recovered and stored in a freezer at −40 °C. The concentration of the dye-labeled peptide was determined as described in the previous section. About 20 nmol of labeled peptide was obtained in each reaction. The MALDI-TOF mass analysis of the labeled peptides was carried out over a mass range reaching at least 800 m/z unit more than that of a singly labeled peptide, and it revealed the presence of a main mass peak corresponding to the singly labeled one for each labeled peptide without any indication of multiple labeling. The structure of the labeled peptide no. 58 is shown on Figure 1.

Determination of p*I* of Labeled Peptides. The p*I* values of the labeled peptides were determined by direct measurement of the pH values of the focused red band formed in a polyacrylamide slab gel IEF. Polyacrylamide gel slabs (5% T, 3% C, 55 × 115 × 1 mm thick) for IEF were prepared on glass plates as described previously using narrow-pH-range Pharmalyte, pH 2.5−5, pH 5−8 ,and pH 8−10.5.[12] The gel plate was placed on the leveled cooling plate of a flat-bed electrophoresis apparatus (model AE-3235D, Atto, Tokyo). Electrodes were placed on the shorter edges of the gel at a distance of 10 cm. A 100-pmol portion of each of the dye-labeled peptides in the collected eluate was applied at the middle of the electrodes using a plastic sample application mask. To estimate the required focusing time (V h cm^{-1}), labeled peptides were applied at two positions near the electrodes. The temperature of the gel under these focusing conditions was adjusted by circulating water controlled at an optimized temperature.[12] Focusing was carried out at a voltage of 250 V for 15 min, at 500 V for an additional 15 min, and then at 1000 V until the focusing reached completion. For the pH range 8−10.5, focusing was carried out in a glovebox filled with humidified nitrogen to exclude any effects of atmospheric carbon dioxide. After electrophoresis, the temperature of the circulating water was raised to 25 °C, and the pH values of the red-focused bands were measured by directly placing the pH sensor of a pH meter (model HM17MX equipped with a Metox pH sensor (model MTX-6101F), TOA Electronics, Tokyo) over them.

Detection Systems. The scanning LIF detector for a capillary is shown schematically in Figure 2. The prototype of this type of scanning fluorescence detector for CIEF was developed in Professor Barry Karger's lab in the Barnett Institute of Northeastern University (Boston) in 1993. The fixed capillary guide employed here to hold the sliding capillary at the correct position in the optical system is an essential element for stable detection. The polyimide coating of a fused silica capillary (18 cm long) was

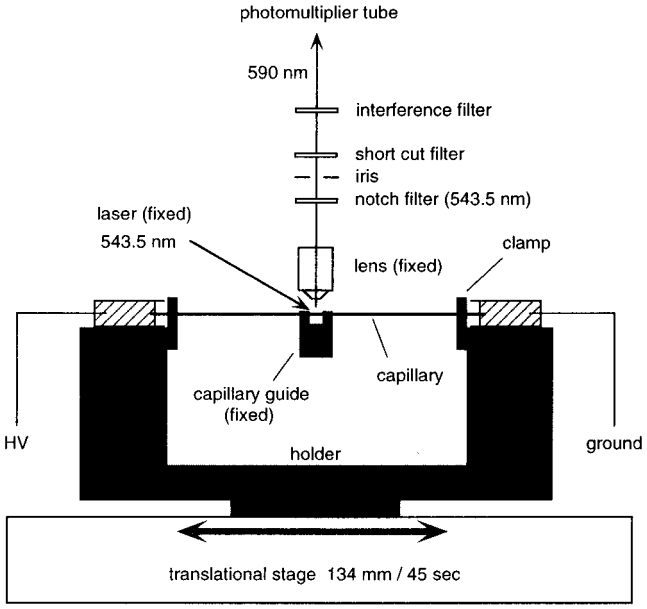

Figure 2. Scanning LIF detector.

removed using a razor blade, leaving the coating at both ends for a distance of 1 cm. The capillary was installed in the holder and attached to an electrical translational stage (model CKR 30-200X, Sigma Koki, Hidaka, Saitama) via two clamps (5 × 15 × 37 mm) that pinched the capillary via a slit that can be tightened by a small bolt in order for the ends to protrude behind the clamps for 5 mm to reach the electrode solution in polypropylene electrode vessels (4 mm i.d., 6 mm o.d., 40 mm long). The vessels were laid on their sides, but the electrode solutions remained in the vessels because of their surface tension. The clamp was attached to the holder by a bolt via a bolt hole opened in the clamp that was larger than the diameter of the bolt to permit the fine adjustment of the clamp in the lateral directions. Simultaneously, the capillary was placed on the groove of the U-shaped lower plate of the capillary guide fixed on a bench via an *xyz* stage and was loosely pressed with another U-shaped plate using a rubber band, thus permitting the capillary to slide in its axial direction. A sheet of Teflon was inserted between the capillary and the upper plate to reduce friction. The holder, the clamps, and the capillary guide were made of ABS resin. The lateral positions of the clamps were adjusted to align the clamping points and the guided points on a line. The output radiation (543.5 nm) from a 1 mW He−Ne laser (model 05-LGR-151-S, Melles Griot, Tokyo) was focused on the capillary at a point between the two guided points at a right angle to the capillary, and the fluorescence was collected at right angles to both the capillary and the laser beam by a 40× objective lens. Fluorescence intensity was measured by use of a photo multiplier tube (model R1387, Hamamatsu Photonics, Hamamatsu, Shizuoka) after filtration with a notch filter (543 nm, Holographic Super Notch Filter, Kaiser Optical Systems, Ann Arbor, MI), a cutoff filter (model SCF-50S-56O, Sigma Koki), and a band-pass filter (590 nm, 30-nm bandwidth, model DIF-BP-3, Nippon Shinkuu Kougaku, Tokyo). The basic design of the optical system was

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based on the published one.[18] Signals were collected using an integrator (model CR4A, Shimadzu, Kyoto). The translational stage was controlled by a stage controller (model MARK-21, Sigma Koki) at a speed of 3 mm/s for scanning and at 30 mm/s for returning. The scanning spanned 134 mm of the central part of the capillary of 180 mm total length.

CIEF. 1. Polyacrylamide-Coated Capillary. The fused-silica capillary (50 μm i.d., 375 μm o.d., 18 cm long, 0.35-μL volume) was coated inside with polyacrylamide according to a published procedure.[21] A working carrier ampholyte solution was prepared by diluting purchased Pharmalyte 40-fold and adding Tween 20 at 0.1% (w/v). The labeled peptide solution in the working carrier ampholyte solution was filled in the capillary installed in the scanning detector. Isoelectric focusing was carried out at field strength of 500 V cm^{-1} for 10−15 min with the electrode solutions of 20 mM phosphoric acid at the anode and 20 mM sodium hydroxide at the cathode. The capillary was scanned over a distance of 13.4 cm from the cathode to the anode for 45 s. All procedures were carried out at room temperature. When needed, acetic acid (0.1% (v/v)) or *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (0.3% (v/v)) or both were added as a spacer between the pH gradient and the anode or cathode electrode solution, respectively.

2. FC-Coated Capillary. The electrophoresis conditions were basically the same as those for the CIEF with the polyacrylamide-coated capillary, as described above, except for the following points.[22] The capillary (μSIL-FC capillaries) was conditioned by filling it with 0.35% methylcellulose for at least 2 h. A working carrier ampholyte solution was prepared by diluting Pharmalyte 40-fold and adding methylcellulose at a final concentration of 0.35%. The electrode solutions were 100 mM H_3PO_4 and 40 mM NaOH, both of which contained 0.35% methylcellulose for the anode and cathode, respectively. Acetic acid or TEMED were added to the working carrier ampholyte solution as described above.

Mass Spectrometry. A matrix-assisted laser desorption ionization time-of-flight mass spectrometer (KRATOS mass spectrometer, KOMPACT MALDI IV, Shimadzu, Kyoto) was used to obtain mass spectra of the labeled peptides. Three matrixes were tested for ionization, that is, sinapinic acid, α-cyano-4-hydroxycinnamic acid, and gentisic acid. Samples dissolved in 50% *n*-propanol were applied onto a layer of the matrix on a sample slide and dried under nitrogen stream. The matrix of α-cyano-4-hydroxycinnamic acid gave good signals for parent ions.

RESULTS AND DISCUSSION

Dye-Labeled Peptide p*I* Markers. Isoelectric-point markers should quickly focus to form sharp bands in a pH gradient. The focusing of small ampholytes in IEF is greatly dependent on the −dz/d(pH) value at its p*I*, that is, $[-dz/d(pH)]_{pH=pI}$,[5,12,23] which represents the dependence of the charge of the ampholyte on pH in the vicinity of its p*I*, where *z* is the net charge of the ampholyte. If we assume the independent ionization of each ionizing groupon a peptide, *z* can be calculated by the following equation

$$z = \sum_i (n_i/(1 + K_i/[H^+])) - \sum_j (n_j/(1 + [H^+]/K_j)) \quad (1)$$

where K_i is the acid dissociation constant (*Ka*) for the conjugate acid of the basic group and K_j is that for the acidic groups and n_i and n_j denote the numbers of such ionizable groups in a particular peptide. The p*I* value can be calculated as the pH where $z = 0$. The following p*Ka* values were used for purposes of calculation. C-terminal carboxyl (3.6), Asp (3.95), Glu (4.45), His (6.45), N-terminal amino (7.6), Tyr (9.8), Lys (10.2) and Arg (12.5). The $[-dz/d(pH)]_{pH=pI}$ value of a peptide was calculated as described before.[5,12] This value becomes large when a peptide contains an ionic group or groups, the p*Ka* value of which is close to the p*I* of the peptide. The contribution of a single ionic group to the $[-dz/d(pH)]_{pH=pI}$ value reaches a maximum of 0.576 when the p*Ka* value is equal to the p*I*. This theory demands the presence of carboxyl groups for acidic p*I* markers, that is, a C-terminal carboxyl group, Asp or Glu residue. On the other hand, for the markers in a neutral pH region, the presence of an N-terminal amino group or a His residue is required, and for basic p*I* markers, the presence of Tyr and Lys residues is essential.[5,12]

On the basis of the above considerations and guided by the calculated p*I* values according to eq 1, 16 peptides (Table 1, no. 44−59) were designed initially, and the synthesis was sent to a commercial peptide-synthesis service. To keep the N-terminal amino groups ionizable, a Cys residue was incorporated into each peptide, and its thiol was labeled with iodoacetylated tetramethylrhodamine (Figure 1). The dye itself is an ampholyte, and its ionization and fluorescence are almost independent of pH from 3 to 10, although some suppression of ionization of the carboxyl group on the phenyl ring of the dye may occur at the acidic extreme below pH 4. The N-terminal Gly was added to increase the yield of peptides in the synthesis. In some peptides, one type of amino acid is doubled or tripled to enhance the contribution of its dissociation on the $[-dz/d(pH)]_{pH=pI}$ value. The structures of the 16 peptides and their relevant values are listed in Table 1. These peptides are identical to the ones designed for p*I* markers for UV detection except for the presence of a glycylcysteine residue that replaces a Trp residue. All of the peptide p*I* markers containing Trp were focused in a satisfactorily short time and can be used as p*I* markers for UV detection.[12,13] On the other hand, in the case of the dye-labeled-peptide p*I* markers, weakly basic ones (no. 44 and 49) and weakly acidic ones (no. 53−55) did not focus well, and their p*I* values could not be determined, despite the fact that their $[-dz/d(pH)]_{pH=pI}$ values are the same as those of peptide p*I* markers for UV detection. To be focused well and for unknown reasons, the dye-labeled markers seem to require a higher $[-dz/d(pH)]_{pH=pI}$ value than those of the Trp-containing markers.

To fill the vacancy of the markers in the weakly acidic and weakly basic pH region, a set of new peptides was designed to increase the number of Glu and His residues for the weakly acidic ones and of Lys and Tyr residues for the weakly basic ones (Table 1, no. 67−73). This attempt was successful for the weakly acidic peptides (no. 70−72) and partially successful for the weakly basic pH region (no. 69 and 73). The peptides for covering the pH range of 8−9 were only slightly soluble (no. 67 and 68). In addition to their sparing solubility, the absence of ionizable groups that have

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Table 1. Summary of p*I* Determination of Tetramethylrhodamine-labeled Peptide p*I* Markers

no.[a]	peptides[b]	p*I* (obs)[c]	SD	*N*[d]	V h cm^{-1}[e]	p*I* (calc)[f]	−dz/d*p*H[g]
46	Gly-Cys-Tyr-Lys-Arg	10.12	0.05	4	113	10.02	1.108
47	Gly-Cys-Tyr-Lys-Lys	9.94	0.02	3	113	9.76	1.490
48	Gly-Cys-Tyr-Tyr-Lys-Lys	9.70	0.02	3	113	9.52	1.725
45	Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys	9.56	0.03	3	113	9.36	1.896
73	Gly-Cys-Lys-Lys-Lys-Tyr-Tyr-Glu-Glu-Tyr-Arg-Tyr-Tyr	9.22	0.02	3	163	9.10	2.134
67	Gly-Cys-Tyr-Tyr-Tyr-Lys-Tyr-Tyr-Tyr-Lys-Tyr-Tyr-Tyr	insoluble				8.88	2.290
49	Gly-Cys-Tyr-Lys	slow focusing			>213	8.62	0.375
74	Lys(Gly)-Cys-Glu-Tyr-Tyr-Lys-Lys-Tyr	8.77	0.04	5	113	8.56	0.867
76	Lys(Gly)-Cys-Glu-Lys(Gly)-Tyr-Tyr-Tyr-Lys-Lys-Tyr-Tyr	8.68	0.05	3	163	8.56	1.308
44	Gly-Cys-Glu-Tyr-Tyr-Lys-Lys	8.71−8.93			>113	8.46	0.519
68	Gly-Cys-Tyr-Tyr-Tyr-His-Tyr-Tyr-His-Tyr-Tyr-Lys	insoluble				8.28	0.880
75	Lys(Gly)-Cys-Lys-Lys(Gly)-Glu	8.21	0.03	3	113	7.90	1.547
69	Gly-Cys-His-His-His-His-His-His-His-His-His	7.74	0.03	4	113	7.66	1.710
50	Gly-Cys-Glu-His-His-His-Arg	7.58	0.03	4	113	7.34	1.229
51	Gly-Cys-Glu-His-Arg	7.38	0.04	4	113	7.04	0.771
52	Gly-Cys-Glu-His-His	6.86	0.04	4	113	6.42	1.311
53	Gly-Cys-Glu-Arg	slow focusing			>113	6.06	0.125
70	Gly-Cys-His-Glu-His-Glu-His-Glu-His	6.18	0.02	3	113	6.02	2.060
54	Gly-Cys-Glu-His	slow focusing			>113	5.48	0.428
71	Gly-Cys-His-Glu-His-Glu-His-Glu-His-Glu	5.53	0.02	3	113	5.46	1.570
55	Gly-Cys-Asp-Asp-His-His	slow focusing			>113	5.24	0.526
72	Gly-Cys-Glu-His-Glu-His-Glu-His-Glu-Lys-Glu	4.99	0.01	3	113	5.02	2.240
56	Gly-Cys-Glu-Glu-His	4.50	0.08	3	113	4.54	1.381
57	Gly-Cys-Asp-Asp-Arg	4.23	0.02	3	113	4.16	1.477
58	Gly-Cys-Glu-Glu	3.99	0.01	3	113	3.82	1.249
59	Gly-Cys-Asp-Asp-Asp	3.64	0.02	3	113	3.38	1.695

[a] The numbers of the peptides used for the mixture of 12 p*I* markers are underlined. [b] The peptides were labeled at the cysteine residues with iodoacetylated tetramethylrhodamine. The amino termini are shown on the left side. Lys(Gly) represents N$^{\epsilon}$-glycyl-L-lysine. L isomers were used, except for glycine. [c] Determined p*I* values. [d] The number of determinations. [e] The labeled peptides were focused within the listed values under the conditions used for p*I* determinations. [f] Calculated p*I* values. [g] Calculated −dz/d*p*H values at pH=p*I*.

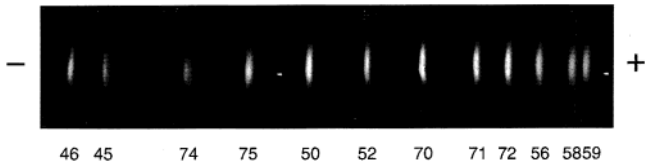

Figure 3. Polyacrylamide-slab-gel IEF of a set of 12 dye-labeled peptide p*I* markers. The mixture of the 12 p*I* markers (No. 46, 45, 74, 75, 50, 52, 70, 71, 72, 56, 58, and 59; 100 pmol each) was separated by IEF on a polyacrylamide-gel slab (5% T, 3% C, 55 × 125 × 1 mm) containing Pharmalyte 3-10 at a 16-times dilution of the purchased solution. Focusing was carried out at 100, 200, 400, and 800 V, for 15 min each, and finally, at 1000 V for 30 min with a distance between the electrodes of 10 cm (87.5 V h cm^{-1}) at 15 °C under nitrogen. The photograph was taken under illumination with UV light using a red optical filter.

a pK_a value in this pH region, except for the N-terminal amino group, made the design of p*I* markers difficult in the form of a linear peptide. To increase the number of N-terminal amino groups, an N$^{\epsilon}$-glycyllysine residue was incorporated. This clearly improved the focusing characteristics of the p*I* markers in the pH region of 8−9 (Table 1, no. 74−76). Finally, we were able to develop 19 fluorescent p*I* markers that focus in a short time covering the pH range of 3.64−10.12 that had a maximum gap of 0.68 pH unit between pH 6.18 and 6.86.

Twelve peptides, no. 46, 45, 74, 75, 50, 52, 70, 71, 72, 56, 58, and 59, were selected to form a mixture of a set of p*I* markers that evenly cover the pH range of 3.64−10.12. IEF on a polyacrylamide-gel slab using Pharmalyte pH 3-10 as a carrier ampholyte showed 12 distinct fluorescent bands under illumination of UV light (Figure 3).

CIEF with Scanning LIF Detector. CIEF of the mixture of the 12 p*I* markers at a concentration of 50 pM each was carried out, and the markers were detected uisng the scanning LIF detector (Figure 4A). All markers focused within 10 min at a field strength of 500 V cm^{-1}. TEMED and acetic acid were included in the carrier ampholyte solution as spacers between the pH gradient and the catholyte and the anolyte, respectively, to bring the entire pH gradient in the scanning range of the capillary. The peaks are higher for the neutral and weakly acidic markers (no. 52, 70-72) and become lower at both pH extremes; however, when the areas of these peaks are compared, no decrease of peak area in the acidic side was observed, but in the basic extreme, it decreased to about two-thirds of that of the neutral-to-acidic markers. Since no change in the ionization state of the dye is expected in the basic pH range, a direct effect of pH on the fluorescence property of the dye is improbable. The reason for this decrease is unknown so far. The plot of the p*I* values of each marker against the scan time, which is proportional to the distance from the cathodic end of the capillary, showed a slightly curved line (Figure 4B). This result demonstrates for the first time the calibration of a wide-range pH gradient formed in a capillary using fluorescence detection.

The polyacrylamide coating was not very stable under the conditions used for IEF, with the wide pH range carrier ampholyte that increases the pH at the cathodic end to values higher than pH 10. This instability may be related to the use of a strong base as an electrode solution. The use of a weak acid and base may improve the stability.[24] Fluorocarbon-coated capillaries, μSIL-FC

(24) Righetti, P. G.; Gelfi, C.; Conti, M. *J. Chromatogr. B* **1997**, *699*, 91−104.

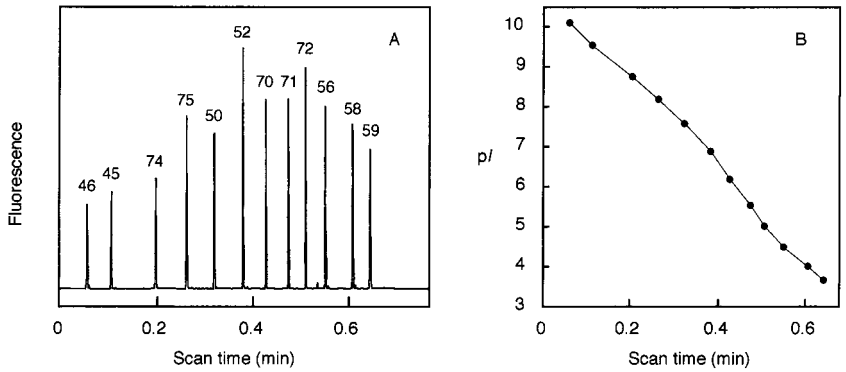

Figure 4. CIEF separation of a mixture of the tetramethylrhodamine-labeled peptide p*I* markers in a polyacrylamide-coated capillary. (A) The 12 p*I* markers in 40-fold-diluted Pharmalyte 3-10, containing 0.1% Tween 20, 0.1% TEMED, and 0.05% acetic acid, each at a concentration of 50 pM (18 amol), were focused for 10 min at a field strength of 500 V cm^{-1} in a polyacrylamide coated capillary (50 μm i.d., 375 μm o.d., 18 cm long). The capillary was scanned for 45 s using the LIF detector from a point 2.3 cm from the cathodic end to a point 2.3 cm from the anodic end. The numbers above the peaks show the p*I* markers used. (B) The pH gradient obtained for the result shown in Figure 4A by plotting the p*I* value of each p*I* marker against scan time.

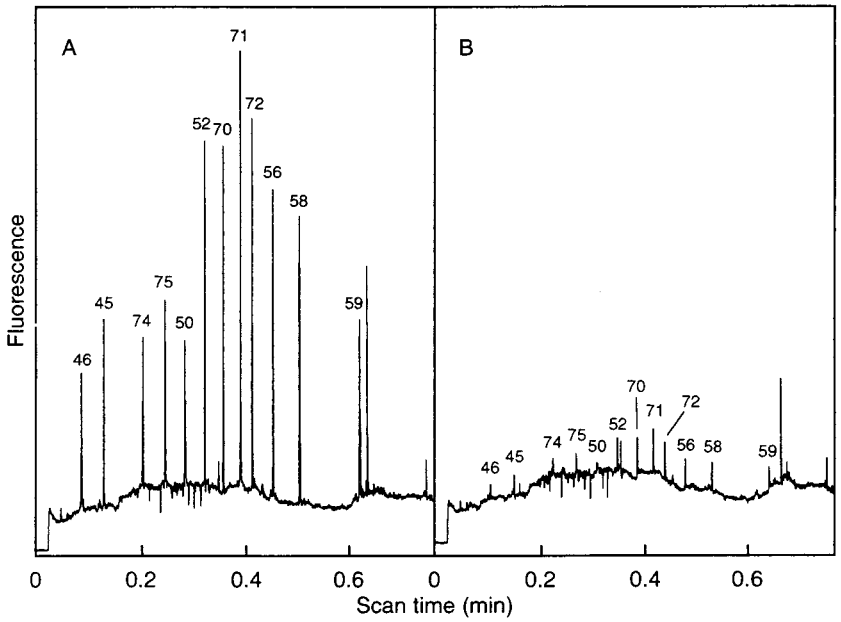

Figure 5. CIEF separation of a mixture of the tetramethylrhodamine-labeled peptide p*I* markers in a fluorocarbon-coated capillary. The 12 p*I* markers in 40-fold-diluted Pharmalyte 3-10 containing 0.35% methylcellulose, 0.3% TEMED, and 0.1% acetic acid were focused for 15 min at a field strength of 500 V cm^{-1} in a μSIL-FC capillary (50 μm i.d., 375 μm o.d., 18 cm long). The capillary was scanned for 45 s using the LIF detector from a point 2.3 cm from the cathodic end to a point 2.3 cm from the anodic end. The concentration of each marker was (A) 1 pM and (B) 100 fM. The numbers above the peaks represent the p*I* markers used.

capillaries, with a dynamic methylcellulose coating show better stability under the LIF conditions. The markers function well under the CIEF conditions with the fluorocarbon-coated capillaries (Figure 5). Even at a concentration of 100 fM, the marker peaks were discernible, although some spike noises became comparably large. No reduction in detection sensitivity was observed for this scanning detector in comparison with the stationary detector using a technique of mobilization of a pH gradient formed in a capillary.[2,5] Stable sliding of the capillary at the detection point seems to be very important for high-sensitivity detection. This can be readily achieved by loosely pressing down the capillary by the upper plate into the grove of the lower plate of the capillary guide and inserting a sheet of Teflon between the upper plate and the capillary.

As an example of the use of the developed markers, the p*I* of a dye-labeled antibody fragment was determined by CIEF. A tetramethylrhodamine-labeled recombinant antibody fragment was focused using the mixture of the 12 markers at a concentration of 10 pM and detected using the LIF scanner (Figure 6). The p*I* value of the labeled protein was estimated to be 5.59 on the basis of the assumption that the pH is proportional to scan time between the two adjacent markers.[13]

Stability and Handling of the Fluorescent p*I* Markers. The fluorescent p*I* markers were found not to be very stable when they were stored improperly. A typical example observed for the acidic markers after storage for 3 days under a fluorescent lamp at room temperature in 1 mM HCl containing 0.1 % Tween 20 is shown in Figure 7B. A new sharply focusing fluorescent peak with a slightly more acidic p*I* value than the original appeared, and each marker focused as a doublet in the CIEF separation. Acidic conditions for storage were selected to prevent microbial or enzymatic degradation, and Tween 20 was added to reduce possible binding of the dye-labeled peptides to the wall of plastic

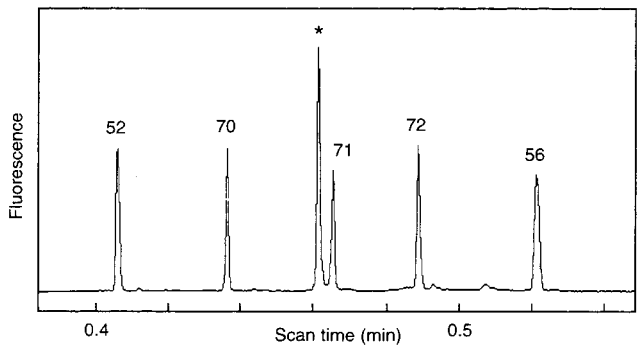

Figure 6. CIEF of a tetramethylrhodamine-labeled antibody fragment with the p*I* markers. A recombinant Fab′ fragment of an antibody labeled with a tetramethylrhodamine dye was mixed with the markers at a concentration of 10 pM each in 40-fold-diluted Pharmalyte 3-10 containing 0.1% Tween 20, 0.1% TEMED, and 0.05% acetic acid. A polyacrylamide-coated capillary (50 μm i.d., 375 μm o.d., 18 cm long) was filled with the solution, and focusing was carried out for 10 min at field strength of 500 V cm^{-1}. At the end of the focusing, the capillary was scanned using the LIF detector. A portion of the electropherogram is shown. The asterisk indicates the peak corresponding to the labeled Fab′; the numbers above the peaks represent the p*I* markers used.

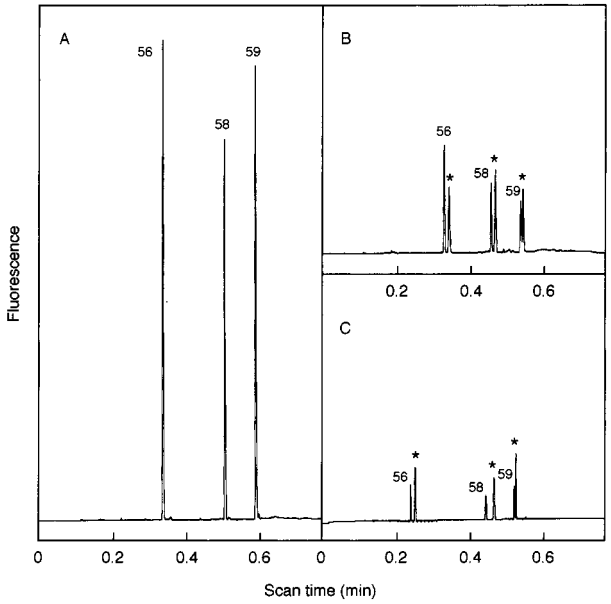

Figure 7. Typical changes observed in the acidic fluorescent pI markers. The original mixture (A) of three fluorescent p*I* markers, no. 56, 58, and 59, each at a concentration of 5 nM, was stored for 3 days in 1 mM HCl containing 0.1% Tween 20 at room temperature under a fluorescent lamp (10 W, 10 cm) (B) or treated with 0.15 mM peracetic acid in 1 mM HCl containing 0.1% Tween 20 at 37 °C for 30 min (C). Aliquots were analyzed by IEF in a polyacrylamide-coated capillary (A and B) or in a μSIL-FC capillary (C) after a 100-fold dilution with each working carrier ampholyte solution using Pharmalyte pH 2.5−5. The asterisks in B and C indicate the newly formed peaks.

vials. Such changes were not observed for the peptide p*I* markers for UV detection, which contained tryptophan instead of the fluorescent-dye-labeled cysteine residue, thus suggesting that the origin of the change can be attributed to the attachment of the dye. The sulfide bond, R−S−R′, which links the dye to the peptides at the cysteine residue (Figure 1), is known to be susceptible to oxidation to a sulfoxide, R−SO−R′.[25] Peracetic acid is an efficient reagent for the oxidation of sulfides.[25] We tested the effect of peracetic acid on the acidic fluorescent p*I* markers and found that the formation of the doublet can be readily reproduced by the addition of 0.15 mM peracetic acid within 30 min at 37 °C (Figure 7C). The irreproducibility of the scan time can be attributable to the conditions or type of capillary that were used.

We also found that the addition of Tween 20 to the storage solution was responsible for the acceleration of the doublet formation. Tween 20 is known to be oxidized to form hydroperoxides[26] and can then oxidize other substances in turn. We suspect that the changes were caused by the oxidation of the sulfide bond between the peptide and the dye moiety to a sulfoxide and that this somehow affected the ionization of the marker to focus at a different position, corresponding to a slightly different pH. To confirm this, tetramethylrhodamine labeled Gly-Cys-Glu-Glu (no. 58) was treated in 50% *n*-propanol with 1 mM peracetic acid at 37 °C for 1 h until the original no. 58 disappeared and was converted completely to the new peak with a slightly low p*I* value in the CIEF analysis. The fluorescent product in the reaction mixture forming a single fluorescent peak was purified by HPLC, as was done for purification of the labeled peptides. The purified fluorescent material was subjected to mass analysis using the MALDI-TOF mass spectrometer. The original labeled Gly-Cys-Glu-Glu gave a parent ion at m/z of 877 with sinapinic acid as the matrix, but the peracetic-acid-treated sample provided a parent ion at m/z of 893, in accordance with the addition of one oxygen atom at the sulfide bond.

The eluted labeled peptides from the HPLC column, at a concentration of about 20 μM in 30−40% acetonitrile containing 0.1% trifluoroacetic acid, can be stored in a freezer at −40 °C for over 3 years without appreciable change. For the dilution of the dye-labeled peptides, 50% *n*-propanol was used as a solvent. The markers, each at 5 nM in this solvent, can be stored for one week at 37 °C in the dark without losing the integrity of the overall separation pattern of the markers. Anhydrous *n*-propanol was tested, but the solubility of the markers is limited in it. Exposure to light is detrimental, and major alteration in the peak pattern of the markers was observed when they were stored for 1 week at room temperature in 50% *n*-propanol under illumination of a fluorescent lamp. The markers should be stored under protection from light and preferably at a low temperature.

Since the LIF-CIEF system is very sensitive and allows the analysis of samples at a level reaching a subpicomolar concentration range, the adsorption of analytes at the wall of a container can be a serious problem if it occurs. The possibility of adsorption of labeled peptides to the wall of a polypropylene sample vial was tested by sequentially transferring the marker solution from one vial to another. A 200-μL portion of a mixed solution of the 12 markers at a concentration of 50 pM each in the working carrier ampholyte solution containing 0.35% methylcellulose and without Tween 20 was placed in a 1.5-mL microcentrifuge tube for 15 min at room temperature. At the end of this period, a portion of the solution was separated by CIEF, and the area of each peak was obtained. The remainder of the solution was transferred to another tube, and the incubation and analysis by CIEF was repeated. No

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reduction in peak area was observed for four transfers of the solution. An identical experiment was carried out for the marker solution in 50% *n*-propanol. Again, no decrease in peak area showing any adsorption of the markers to the wall of the tube was observed.

CONCLUSIONS

Markers have been developed on the basis of a commercially available peptide synthesis that uses amino acids that normally occur in peptides and proteins. As shown above, the distribution of pK_a values of ionic groups in such amino acids is not evenly spaced over the pH range used in IEF. If the scope of amino acids is expanded to include artificial ones, a more appropriate choice of dissociation groups becomes available for the design of p*I* markers that have good focusing characteristics at the desired isoelectric points. We were then able to obtain markers with simpler structures. Although the range of applications of CIEF-LIF is still limited, the advantage of CIEF for low-concentration samples is obvious. Since monitoring of the form of a pH gradient is the fundamental basis for the application of IEF, the development of these fluorescent markers should stimulate the development of the new applications of CIEF with LIF detection.

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