We report a system for automated protein analysis. In the system, proteins are labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde, which reacts with lysine residues and creates a highly fluorescent product. These labeled proteins are analyzed by submicellar capillary electrophoresis at pH 7.5 to perform a first dimension separation. Once the first components migrate from the capillary, a fraction is transferred to a second dimension capillary, where electrophoresis is performed at pH 11.1 to further separate the proteins. Laser-induced fluorescence is used as an ultrasensitive detector of the separated proteins. Successive fractions are transferred from the first dimension capillary to the second dimension capillary for further separation to generate, in serial fashion, a two-dimensional electropherogram. The transfer of fractions is computer-controlled; there is no operator intervention once the sample has been injected. Zeptomoles of labeled proteins are detected, providing exquisite sensitivity.

The resolution of complex samples into components requires sophisticated technology. Most separation techniques are capable of resolving, at most, several dozen components. Cal Giddings (1) recognized that the combination of two separation techniques is important in the resolution of complex mixtures. If the two separation techniques are based on unrelated characteristics of the sample, then the number of resolution elements is given by the product of the resolution elements of both separation steps. For example, isoelectric focusing and SDS-polyacrylamide gel electrophoresis can, individually, resolve ~50 components in a protein sample. Their combination, in two-dimensional electrophoresis, can resolve several thousand components (2).

Unfortunately, classic two-dimensional electrophoresis requires manual manipulation of the sample. These manipulations, although reasonable for occasional use, are very tedious when performed in large-scale protein analysis projects. Furthermore, detection sensitivity is limited, and relatively large amounts of sample are required to detect weakly expressed components.

Column-switching technology is an alternative means of performing two-dimensional separations and is commonly used in chromatographic resolution of complex samples. Most simply, a fraction containing the compound of interest is captured as it elutes from the first column and is transferred to another chromatographic column for additional resolution; fractionation is repeated with different chromatographic methods to achieve the desired purity.

In multidimensional chromatography, also known as comprehensive chromatography, a second column is used to sequentially separate all fractions from the first column. These two-dimensional techniques are particularly useful when characterizing extremely complex samples. An early report used two successive chromatographic steps to purify peptides generated from the proteolytic digest of a human immunoglobulin (3).

Jorgenson and others (4–12) have developed elegant multicolumn separations for proteins and peptides. These systems rely on various combinations of size exclusion chromatography, reversed-phase chromatography, and zone electrophoresis to characterize amines, peptides, and proteins. In the most sophisticated version, a mass spectrometer is used to identify components separated by a coupled ion exchange/reversed-phase or size-exclusion/reversed-phase chromatography system (8–9).

In the experiments of Jorgenson and co-workers (4–12) (Fig. 1) a portion of each peak that elutes from a microbore chromatography column is sequentially injected into an electrophoresis capillary, where overlapping components are resolved. Because electrophoresis is faster than the elution time of a chromatographic peak, a portion of each peak from the chromatography column is sampled by the electrophoresis capillary. A two-dimensional separation is reconstructed by plotting the electrophoresis separations next to each other. The appearance of the plot is quite similar to a classic two-dimensional electropherogram, although it is generated by combining liquid chromatography with capillary electrophoresis.

Sequential separation offers two advantages over conventional two-dimensional electrophoresis, where components are separated simultaneously. First, because components are
detected sequentially, a sensitive detector can be incorporated into the instrument. Second, the separation is automated; once the sample is injected, there is no further operator intervention. However, sequential separation is usually slower than parallel separation.

The interface between the two columns is key to the performance of the system. Jorgenson and co-workers (11, 12) have demonstrated an elegant flow-gated interface to couple an HPLC column with a capillary electrophoresis column. This interface uses a flow of buffer to control injections of chromatographic fractions into a capillary zone electrophoresis column. The effluent continually migrates from the HPLC capillary and is swept to waste by the cross-flow in the interface. To inject a fraction into the electrophoresis capillary, the cross-flow of buffer is halted, and a plug of HPLC effluent forms in the interface. Potential is applied from the buffer vial across the electrophoresis capillary to the detection end of the capillary, which injects the HPLC effluent into the capillary. The buffer flow is reapplied to the interface, terminating the injection. The electric field remains applied across the capillary to separate the injected components. Unfortunately, to avoid continuous injection of the HPLC components during the electrophoresis step, only a small portion of each peak is injected into the electrophoresis column; most of the HPLC effluent is directed to waste.

We have reported the use of capillary electrophoresis for the ultrasensitive analysis of proteins (13, 14). Our systems rely on the fluorogenic reagent 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) to convert lysine residues to highly fluorescent product. This reagent is non-fluorescent until it reacts with a primary amine in the presence of a nucleophile. The use of a fluorogenic reagent reduces the background signal significantly compared with the use of conventional fluorescent reagents.

When coupled with a high sensitivity laser-induced fluorescence detector, detection limits of a few zeptomoles are routinely achieved. We have more recently demonstrated the separation of proteins from a single cancer cell using both submicellar and SDS-DALT electrophoresis, where the latter term refers to size-based electrophoresis of proteins in a sieving matrix (15, 16).

Unfortunately, our one-dimensional separations are unable to resolve the large number of components present in a complex cellular lysate. In this paper, we report the development of a fully automated, two-dimensional electrophoresis system to further resolve these components. Our system differs from that of Jorgenson and co-workers (4–12) in three important respects. First, we use capillary electrophoresis in both dimensions of the separation, which eliminates the use of chromatography, with its associated pumps, valves, and large sample volume. Second, we transfer the entire fraction from the first capillary to the second, eliminating the loss of sample in the flow-gated interface. Third, we use an extremely high sensitivity fluorescence detector, which allows us to monitor zeptomoles of proteins.

**EXPERIMENTAL PROCEDURES**

Reagents—The labeling reagent FQ and cyanide are from Molecular Probes. Water is deionized and distilled (Barnstead). All other reagents are from Sigma.

Sample—Protein extracts are prepared from HT29 human adenocarcinoma cells (14). Roughly 10⁸ cells are lysed by sonication and labeled with 100 nmol of FQ in the presence of 2.5 mM potassium cyanide at 65 °C for 5 min. The FQ-labeled cell extract is diluted 50-fold in 10 mM phosphate buffer, pH 7, and stored on ice (15).

**Electrophoresis Buffers**—A 10 mM HEPES and 5 mM SDS buffer, pH 7.5, is used for the first dimension separation whereas the second dimension separation buffer is 40 mM CAPS and 5 mM SDS, pH 11.1.

**Instrument**—Fig. 2 presents a schematic diagram of the fully automated two-dimensional capillary electrophoresis instrument. Two-dimensional electrophoresis is driven by two 0–30,000-V power supplies (Ultra Volt) that are controlled by a Macintosh II computer and driven by software written in Labview (National Instruments).

Separation is performed in two 40-cm-long, 50-μm-inner diameter, 138-μm outer diameter polyamide-coated fused silica capillaries (Polymicro). The polyamide coating is burned away from the detector end with a gentle flame. Buffer 2 (CAPS) inlet and outlet capillaries have 145-μm inner diameters/367-μm outer diameters with lengths of 6 and 15 cm. The running Buffer 1 (HEPES), running Buffer 2, and detector buffer reservoirs are kept at equal heights to prevent siphoning through the capillary during the experiment.

The black square in Fig. 2 represents a modified version of Jorgenson’s interface that aligns the two separating capillaries and the two waste capillaries. The machine shop at the Chemistry Department, University of Alberta drilled two 370-μm diameter holes in a piece of clear Lexan fitted in a cross (Valco). Chamfered holes were also drilled to hold ferrule fittings. Before assembly, the interface is rinsed with distilled water to remove any particles and air trapped in the cross. With the aid of a microscope, each of the four capillaries is threaded through 1/16-inch sleeves and carefully lined in position. The capillaries are held in place by tightening the ferrules in the Valco cross.

The first dimension capillary inlet is placed in the injection buffer.
reservoir whereas the second dimension capillary outlet is connected into the sheath-flow cuvette. The 6-cm-long Buffer 2 outlet capillary is placed in the Buffer 2 reservoir whereas the 15-cm-long Buffer 2 inlet capillary is connected to a wash-bottle located above the interface to allow gravity flow. Flow in the Buffer 2 inlet is controlled using a low pressure shut-off valve (Upchurch).

Prior to a separation, the capillaries are washed by purging with 0.25 M NaOH for 5 min with nitrogen gas. To ensure flow through both capillaries, the Buffer 2 inlet valve remains closed, and the Buffer 2 outlet capillary is plugged.

The second dimension capillary is filled with running Buffer 2 by pressure, whereas both the Buffer 2 inlet and outlet are plugged. The first dimension capillary is purged with Buffer 1 whereas the Buffer 2 inlet valve is opened, and the Buffer 2 outlet capillary is unplugged.

Sample injection is done hydrodynamically by applying a brief pulse of vacuum to the sheath-flow cuvette while the valve is off, and the waste capillary outlet is plugged (17). After injection, a preliminary separation occurs in the first dimension before fractions are transferred to the second dimension capillary. Here, 10 kV is applied to power supply 1, and 0 kV is applied to power supply 2 for 6–7 min while the waste valve remains opened.

Following the preliminary separation, the waste valve is closed, and second dimension separation cycles begin. A fraction from the first dimension is moved into the second dimension capillary by applying 20 kV with power supply 1 and 10 kV with power supply 2 for 2–10 s. Once the fraction is transferred, the second dimension separation is performed by applying 10 kV to power supply 2. Power supply 1 is adjusted to ensure the same potential is applied to the inlet and outlet of capillary 1, which prevents flow of sample in the first capillary during the second dimension separation. The cycles of fraction transfer and second dimension separation are repeated under computer control.

Laser induced fluorescence detection with a sheath-flow cuvette is used to monitor the separated fluorescently labeled proteins (18, 19). The sample stream from the second dimension capillary is focused in the sheath-flow cuvette and illuminated with an argon-ion laser beam, λ = 488 nm, focused with a 25-mm focal-length lens.

RESULTS

To optimize each separation, the two-dimensional electrophoresis instrument was first configured to operate in a one-dimensional mode. Both capillaries were filled with the same buffer. The injection end of the capillary was held at +20 kV, the interface at +10 kV, and the detector at ground. In this way, the sample is separated at 20-kV potential across an 80-cm-long capillary.

Fig. 3 presents two capillary electropherograms generated from whole-cell lysates of the HT29 cells. The top separation was performed in a pH 7.5 buffer, and the bottom dimension was performed in a pH 11.1 buffer. Roughly two dozen com-
ponents are observed in the low pH separation whereas nearly four dozen components are resolved in the high pH separation. The peaks generate between 50,000 and 100,000 theoretical plates, which demonstrates that movement of the sample across the interface does not introduce significant band broadening.

The instrument was then configured to operate in a two-dimensional mode. Samples were subjected to electrophoresis in the first capillary at pH 7.5, and a fraction was transferred for further separation at pH 11.1. This successive transfer and separation of fractions was repeated 100 times. Fig. 4 presents a two-dimensional electropherogram generated with the sequential application of these two electrophoresis methods.

The top trace was obtained by summing the data along each column of the two-dimensional data and reproduces the electropherogram expected if the sample was simply separated with the second dimension capillary. The left-hand trace was obtained by adding the data along each row of the two-dimensional data and corresponds to the trace expected from a separation generated by the first dimension capillary.

Fig. 5 is an overexposed, close-up image of the region between 1 and 22 fractions from the first capillary and 150–300 s in the second dimension. Fig. 6 is a waterfall image of the same region.

**DISCUSSION**

**Submicellar Separations**—There is an important artifact observed in capillary electrophoretic separations of fluorescently labeled proteins. The most common labeling reagents target the ε-amine group of lysine residues. It has proven difficult to exhaustively label all lysine residues, and a population of reaction products is produced. If there are \( n \) lysine residues, there are \( 2^n - 1 \) possible reaction products (20). For example, ovalbumin has 20 lysine residues; there are 1,048,575 possible fluorescent reaction products from the labeling reaction. Each of these products can have a different electrophoretic mobility, leading to a complex electropherogram with poor separation efficiency.

We have found that the use of FQ as a labeling reagent and the use of an anionic surfactant at submicellar concentrations generates very high resolution electropherograms of proteins, collapsing the labeling reaction products into a peak with as
high as 100,000 theoretical plates (14). The FQ reagent reacts with primary amines to create a neutral product. We believe that the anionic surfactant ion pairs with unreacted lysine residues, also creating a neutral product, so that the net charge on the protein is independent of the extent of labeling.

It is undesirable to use higher concentrations of surfactant. At high concentrations, SDS binds to proteins at a constant ratio, leading to a uniform size-to-charge ratio and a very narrow separation window in the absence of a sieving medium during electrophoresis.

Protein adsorption on the inner wall of the separation capillary remains a significant challenge. We have demonstrated that proteins remain adsorbed to the capillary wall in single-cell analysis, and adsorptive losses are likely to occur in the analysis of protein extracts (17). In this experiment, we flush the capillary between runs to remove adsorbed proteins that might interfere with subsequent separations. We have demonstrated that modified acrylamide coatings are useful in reducing protein adsorption during capillary electrophoresis (21). Such coatings will be required for the analysis of highly basic and highly hydrophobic proteins, which were likely lost during our analyses.

Two-dimensional Electrophoresis—It is important that the two separation mechanisms are uncorrelated so components that are not resolved in the first dimension are separated in the second. We systematically investigated the separation of protein homogenates performed in free-solution electrophoresis at pH levels ranging from 11.5 to 6.0. In all cases, separation efficiency was high, with typical separation efficiency of 100,000 plates. Although there were variations in electrophoresis with pH levels and ionic strength, the separation profile was quite consistent from pH 11.5 to 8. The patterns changed significantly at lower pH levels, presumably because of the protonation of cysteine and histidine side chains. We chose the pH 7.5 and 11.1 buffers for this proof-of-principle experiment, because the two buffers generate quite different separation profiles for the cellular lysate sample. We are working to perform SDS-DALT electrophoresis in the first dimension and submicellar electrophoresis in the second dimension, which should produce improved two-dimensional separation.

Generation of the two-dimensional electropherogram can be time-consuming. Careful attention to the characteristics of the one-dimensional separations was used to reduce the time necessary to generate the two-dimensional electropherogram. In Fig. 3, the first component migrated from the pH 7.5 capillary at 8 min. This electropherogram was generated with the instrument operating as a one-dimensional electrophoresis instrument with an 80-cm effective capillary length. It took 4 min for the first component to pass through the first 40-cm-long capillary and to reach the interface. When generating the two-dimensional electropherogram, we performed a preliminary separation of the sample by applying an electric field to the first dimension capillary continuously for 4 min.

Potential must be applied to the first capillary for an additional 5 min to complete the separation. This potential is only applied during the period when fractions are eluted from the first capillary and transferred to the second. The transfer period is 6 s, and about 50 such transfers are required to elute all fractions from the first capillary and transfer them to the second.

Roughly 24 min are required to separate the sample in the pH 11.1 buffer, using the 80-cm effective capillary length in Fig. 3. It takes about 12 min for the fastest component to reach the detector, and the separation window lasts another 12 min until the slowest component migrates from the capillary. Of course, it takes about 6 min for the fastest component to pass through the 40-cm-long second dimension capillary, and the separation window is also 6 min. To decrease the analysis time, we inject fractions into the second capillary at 6-min intervals. As a result, two fractions undergo separation in the second dimension capillary. The transfer times are chosen so that the slowest component of the first fraction does not interfere with the fastest component of the second fraction.

The overall analysis time is long. About 8 h are required to generate the two-dimensional electropherogram of Fig. 4. However, this separation is performed without operator intervention or supervision. We have demonstrated that a multiple capillary sheath-flow cuvette is an efficient detector in high throughput DNA sequencing (22–24). We plan to modify the two-dimensional electrophoresis instrument so that 96 samples can be analyzed in parallel. A single instrument could analyze 300 samples per day with virtually no operator intervention.

The instrument operates with exquisite sensitivity. Our protein sample contained the extract from roughly 10⁶ HT29 cancer cells, which were prepared in a 100-μl volume. The sample was labeled and diluted 50-fold before analysis. For analysis, an ~5-nl aliquot of labeled proteins was injected into the capillary, and the amount of protein injected is equivalent to that contained in a single cell. The detection limit of the instrument is a few zeptomoles of labeled protein, and the faintest components in Fig. 5 are present at a few thousand copies.

It is important to recognize one limitation of this system. The fluorescence detector, although providing exquisite sensitivity, provides very little information on the identity of the protein. An interesting alternative system would employ a mass spectrometer as a detector. Although lacking the sensitivity of the fluorescence detector, the mass spectrometer could provide sufficient information to identify the proteins.

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Automated Two-dimensional Proteomics

REFERENCES