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Determination of insulin in single pancreatic cells by capillary electrophoresis and laser-induced native fluorescence

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Abstract

Development of a method for the determination of insulin based on capillary electrophoresis with laser-induced native fluorescence detection is described. Under optimal conditions, insulin as low as 73 amol can be detected with a good signal-to-noise ratio ($S/N=10$ peak-to-peak). Application of this method for the determination of insulin content in single cells from the insulin-secreting cell lines RINm5F and βTC3 is demonstrated. Non-bonded poly(ethylene oxide)-coated and bare capillaries are evaluated for this purpose, with the latter found to be more suitable for single-cell analysis.

Keywords: Insulin

1. Introduction

Insulin is an important hormone present in pancreatic β-cells for regulating glucose metabolism. An increase in the blood glucose concentration will stimulate the secretion of insulin from the pancreatic β-cells. The secreted insulin is transported to the liver through blood circulation and stimulates the destruction of glucose by the liver to produce glycogen, so as to maintain glucose levels within the normal range. Deficiency of insulin at the cellular level affects not only the glucose concentrations, but also influences the overall endocrine arena within which it is normally active [1]. Spontaneous diabetes is characterized by a nearly total deficiency of insulin (Type I) or improper insulin secretion (Type II) of the β-cells. The determination of insulin at the single-cell level may thus generate unique information about the variation in insulin content and secretion behavior among cells and the possible relationships between them. This will enhance our understanding of diabetes and possibly facilitate the development of better therapy.

Traditionally, insulin is determined by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [2–5]. Although these methods are very sensitive, the procedures are tedious and slow. More importantly, they cannot be applied to extremely small samples such as a single cell. Capillary electrophoresis (CE), with its extremely small sample volume and high separation efficiency, has proven to be a powerful tool for single-cell analysis [6–23]. Recently, CE-based immunoassay [24,25] and chemically modified electrodes [26] have been used to determine insulin content and to monitor secretion from single islets and β-cells. Most of the methods for insulin assay so far are based on the indirect detection principle. However, the simplest

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detection scheme for single-cell analysis by CE is to measure the native properties of the analytes. Therefore, electrochemical- [6,9–12] and laser-induced native fluorescence (LINF) detection [15,22] have been used. Here, we describe the use of CE–LINF excited at 275 nm to determine the amounts of insulin in individual insulin-secreting β-cells by measuring native fluorescence.

2. Experimental

2.1. CE apparatus

The CE set-up is laboratory-made and is similar to that described previously [14]. Briefly, a high-voltage power supply (Series MJ, 0–30 kV; Glassman High Voltage, Whitehouse Station, NJ, USA) was used to drive the electrophoresis. A 30-μm I.D.–360 μm O.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used for the separation. The total length was 60 cm and the detection window was 43 cm from the injection end. For single-cell measurements, a bare capillary was used after rinsing with 0.1 M NaOH for 5 min and equilibrating with running buffer for 10 min. For lysed-cell measurements, a non-bonded poly(ethylene oxide) (PEO)-coated capillary [27] was used. The capillary was treated with 0.1 M HCl and 0.2% PEO (in 0.1 M HCl) for 5 min, respectively, before finally being flushed with the running buffer. The entire electrophoresis and detection system was enclosed in a sheet-metal box with HV interlocks. The buffer reservoir at the high-voltage end was enclosed in a plexiglass box.

The 275 nm line from an Ar ion laser (Model 2045, Spectra Physics, Mountain View, CA, USA) was isolated from other lines with an external prism and focused with a 1-cm focal length quartz lens onto the detection window of the capillary. One WG-305 and one UG-11 filter (Melles Griot, Irvine, CA, USA) were used to block the scattered light and room light from reaching the photomultiplier tube (PMT). A low-pass (1 Hz) filter was employed to limit the output signal bandwidth. Data were collected at 5 Hz by a 24-bit A/D conversion interface (ChromPerfect, Justice Innovation, Palo Alto, CA, USA). The data were stored in an IBM/PC-compatible computer.

2.2. Methods

Because of the difficulties in isolating large numbers of highly purified pancreatic β-cells, insulin-producing β-cell lines are usually used as a model for wild type pancreatic β-cells [28]. Rat RINm5F cells [29] and mouse βTC3 cells [30] were generous gifts from Dr. W. Hsu, College of Veterinary Medicine, Iowa State University. Before analysis, the cells were washed five times with 5 ml portions of balanced salt solution. Hydrodynamic injection similar to that described in Ref. [14] was used to inject a single cell into the end of the capillary. Once the cell adheres to the capillary wall, 0.05% SDS in a 20-mM Tricine solution (pH 8.5) was injected for 3 s at 30 kV to lyse the cell. Separations were performed at 30 kV. Quantitation is achieved by measuring the peak areas and comparing them with those obtained for calibration runs with standard solutions of insulin between cell injections.

2.3. Reagents

A balanced salt solution was composed of 136 mM NaCl, 5.4 mM KCl, 0.5 mM NaH_2PO_4, 0.34 mM Na_2HPO_4, 0.8 mM $MgSO_4$ and 1.3 mM $CaCl_2$ and adjusted to pH 7.4 with NaOH. Bovine insulin and Tricine were purchased from Sigma (St. Louis, MO, USA). PEO of M_r 8 000 000 was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were purchased from Fisher (Fair Lawn, NJ, USA).

3. Results and discussion

3.1. Native fluorescence of insulin

The native fluorescence of insulin arises mainly from its tyrosyl residues. Fluorimetry measurement shows an excitation maximum at 275 nm and an emission maximum at 305 nm. The positions of the excitation and emission maxima change very little with environment [31]. Thus, the Ar ion laser at 275 nm is suitable for inducing the native fluorescence of insulin. Fluorescence intensities of insulin in differ-

ent media were measured off-line with a fluorimeter. Insulin ($5 \cdot 10^{-6}$ M) was dissolved in various media and the fluorescence intensities were measured in a 1-cm cell with 275 nm excitation. The fluorescence intensities obtained at about 305 nm were normalized to that in water and are depicted in Fig. 1. Under the conditions tested, the fluorescence efficiencies change by less than one fourth. However, in strong acidic solutions (pH<2), protonation lowers the insulin fluorescence. In alkaline solutions (pH>9), the phenolic groups of the tyrosyl residues will ionize, causing a loss of fluorescence [31].

3.2. Separation and detection

One of the major concerns in the separation of proteins by CE is the interactions that occur with the inner surface of the fused-silica capillaries, which will cause peak broadening and a change of the electroosmotic flow (EOF) rate. We have recently demonstrated the application of non-bonded PEO-coated capillaries in protein separation [27]. Here, a PEO-coated capillary is also tested for insulin separation. The output of the photomultiplier tube (PMT) is negative. Therefore, the fluorescence peak appears as a negative one. The signal, background and noise levels were dependent on alignment and the power of the laser. There are always some variations every time we make an adjustment to the set-up. Therefore, only relative signal levels are plotted. Fig. 2A is the electropherogram of insulin in 50 mM citric acid (pH 2.3). A nice sharp peak is obtained in a reasonably short period of time. With the PEO-coated capillary, 73 amol of insulin can be detected with a good signal-to-noise ratio (S/N=10, peak-to-peak) (Fig. 2B). The advantage of a PEO-coated capillary is that the EOF is very small. Also, there is less interference from negatively charged components, since they will move in the opposite direction and will not be detected.

Insulin is an acidic protein with a pI value of 5.7 [32]. When the running buffer is basic, both insulin and the capillary wall are negatively charged. There should be no adsorptive interaction between insulin

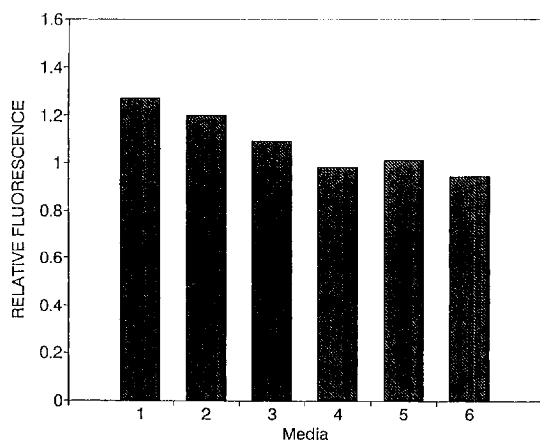

Fig. 1. Relative fluorescence intensities of insulin in various media normalized to that in H_2O. (1) 50 mM citric acid (pH 2.3); (2) 50 mM acetic acid (pH 3.0); (3) 20 mM NaH_2PO_4 (pH 4.8); (4) 20 mM boric acid (pH 5.6); (5) 20 mM Na_2HPO_4–NaH_2PO_4 (pH 6.9); (6) 20 mM sodium borate (pH 9.0).

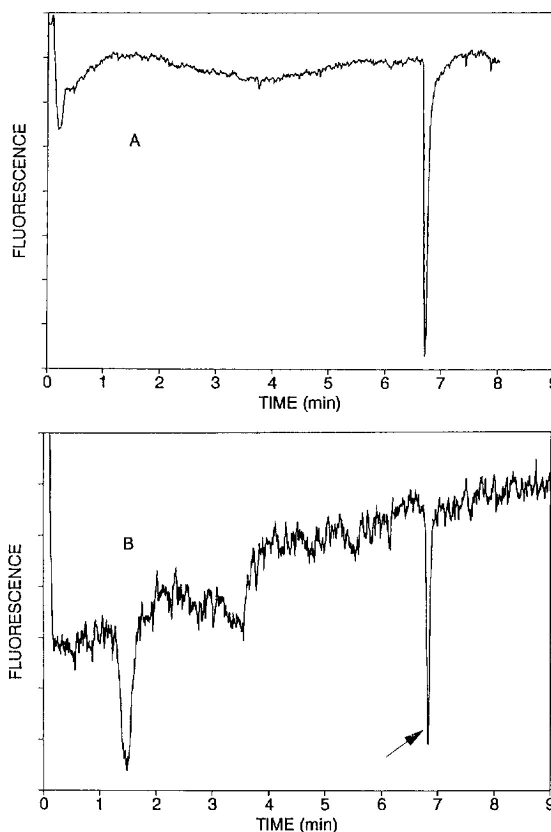

Fig. 2. Electropherograms of insulin separated in a PEO-coated capillary; +30 kV was applied to the injection end. Running buffer, 50 mM citric acid; (A) 730 amol of insulin were injected; (B) 73 amol of insulin were injected.

and the capillary wall. At the same time, the native fluorescence of insulin is still quite strong in moderately basic solutions. Separation of insulin with a bare capillary in basic running buffer is therefore evaluated. Fig. 3A is the electropherogram of insulin in 20 m*M* Tricine (pH 8.5). The separation is even faster and the peak is even sharper. As a result, the detection limit is still similar to that in citric acid (Fig. 3B), despite the lower fluorescence efficiency.

3.3. Single-cell analysis

Fig. 4 shows the electropherogram of lysed RINm5F cells (1 μl of cells lysed ultrasonically in 200 μl of water) in a PEO-coated capillary. Since the average size of the cells is 20 μm, the number of cells corresponding to 1 μl of cells is around $2.4 \cdot 10^5$. Based on this assumption and the volume of the sample injected into the capillary, the injected amount was derived from about 1.7 cells. The average amount of insulin in single RINm5F cells is thus estimated to be 140 amol. In another batch of RINm5F cells, the average value was found to be 227 amol/cell. Individual normal β-cells contain 2.5 to 10 fmol of insulin [26]. Since RINm5F cells are induced tumor cells, the cellular insulin contents are usually 0.5 to 2% that of normal cells [28]. This agrees with our results.

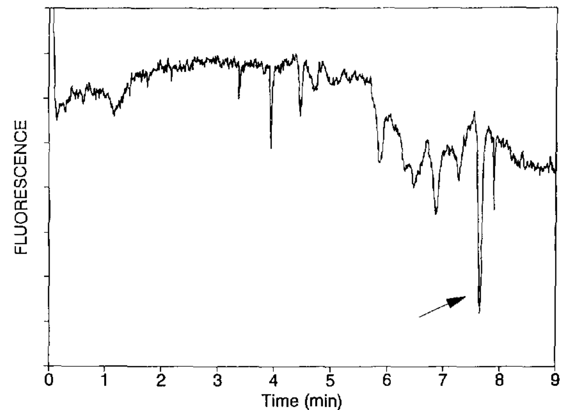

Fig. 4. Electropherogram of insulin in lysed RINm5F cells. Conditions are the same as in Fig. 2.

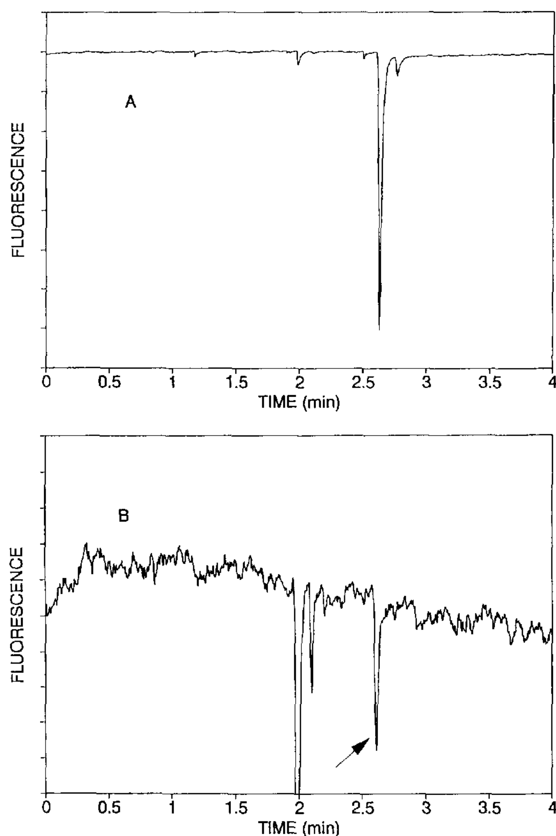

Fig. 3. Electropherograms of insulin separated in a bare capillary; +30 kV was applied to the injection end. Running buffer, 20 m*M* Tricine (pH 8.5); (A) 3.6 fmol of insulin were injected; (B) 73 amol of insulin were injected.

Several problems arise when using PEO-coated capillaries for single-cell analysis. First, unlike red blood cells, which are readily lysed by osmotic pressure in a few seconds, the induced tumor cells (RINm5F and βTC3) are very rugged and will not lyse in a reasonably short time (e.g., a few minutes) in the running buffer to release all their cellular components. Second, the injected cell does not adhere well to the capillary wall, so that specialized lysing techniques, such as a tesla coil [33] or subsequent injection of lysing reagent [21], cannot be easily applied. For these reasons, a bare capillary is employed for single-cell analysis even though the PEO-coated capillary is better for separation.

Cell injection is performed under a 100× microscope. Once the cell is injected into the bare capillary, it will stick to the wall. After cell injection, the capillary containing the cell is carefully removed from the microscope and placed in a reservoir

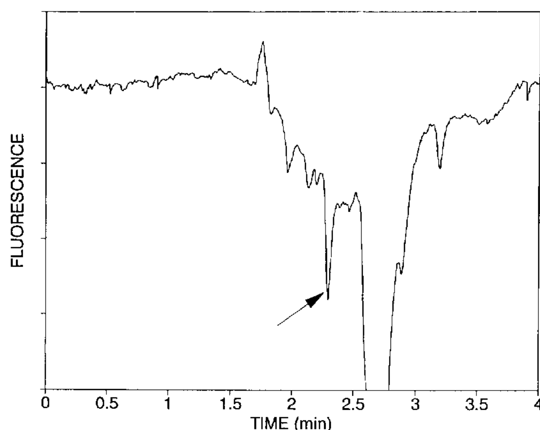

Fig. 5. Electropherogram of insulin in a single βTC3 cell. Conditions are the same as in Fig. 2.

containing 0.05% SDS and running buffer. SDS is electrophoretically injected into the capillary to lyse the cell. Then, the capillary is returned to the running buffer and the separation is initiated. Fig. 5 shows the electropherogram of the contents of a single βTC3 cell. A good signal-to-noise ratio ($S/N>10$) is obtained. A large peak due to injected SDS is evident around 2.7 min. However, it is well separated from the insulin peak.

The insulin contents in thirteen individual βTC3 cells were analyzed consecutively and the results are shown in Fig. 6. The average insulin content in these cells is 1.55±0.74 fmol, which is much higher than that found in RINm5F cells. This not surprising since the insulin content in βTC3 cells has been reported to be 20–30% of that present in normal cells [34]. In another batch of βTC3 cells, nine cells were analyzed. After discarding an outlier by the Q-test, the average insulin content was found to be 1.89±1.03 fmol. The variations in the content of insulin among cells are obvious (R.S.D.s are 48 and 54%, respectively). Another phenomenon in Fig. 6 is that there is a decreasing trend in the insulin content as the experiment proceeds. Each single-cell analysis takes about 10 min. We also run standard insulin solutions for calibration in between analysis runs. After several runs, the capillary also needs to be flushed with NaOH and re-equilibrated using the running buffer. Therefore, the total analysis time for thirteen cells was about 4 h. During this period, the cells may undergo basal release of insulin or may even be partially lysed, resulting in some loss of intracellular insulin.

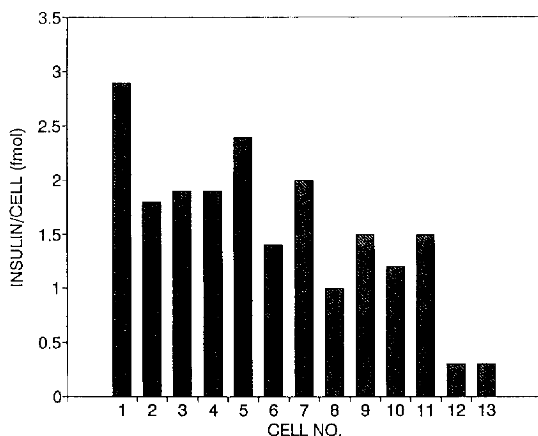

Fig. 6. Insulin content in individual βTC3 cells.

Acknowledgments

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