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Analysis of amino acids in individual wheat embryonic protoplast

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Summary. Amino acids analysis in single wheat embryonic protoplast was performed using capillary electrophoresis equipped with laser-induced fluorescence (CE-LIF), combination with tissue culture technique. Reagent fluorescein isothiocyanate (FITC) was introduced into living protoplasts by electroporation for intracellular derivatization. A special osmotic buffer (0.6 mol/L mannitol, 5 mmol/L $CaCl_2$) was used to keep the osmotic balance of embryonic protoplasts during the protoplasts derivatization. After completion of the derivatization reaction in the protoplasts, a single protoplast was drawn into the capillary tip by electroosmotic flow. Then a 0.1 M NaOH lysing solution was injected by diffusion. The derivatized amino acids were separated by capillary electrophoresis and detected by laser-induced fluorescence detection after the protoplast was lysed Nine amino acids were quantitatively and qualitatively determined and compared in lysate and single protoplast of wheat embryonic cells respectively, with mean concentrations of amino acids ranging from 2.68×10^{-5} mol/L to 18.18×10^{-5} mol/L in single protoplast.

Keywords: Wheat embryonic protoplast – Amino acid – Capillary electrophoresis – Electroporation – Laser-induced fluorescence – Single-protoplast analysis

Introduction

It is known that CE has some advantages over other methods, e.g. fast and efficient operation, lower cost and biocompatible environment. In addition, only a small amount of sample is needed when CE combined with laser-induced fluorescence (LIF) detection, which has higher sensitivity. Many chemical contents of different cells have been detected by CE-LIF (Smith, 1999; Poinsot et al., 2003; Zhang et al., 2005). It is more and more widely used in clinical studies, neurochemistry, food and agricultural analysis (Poinsot et al., 2003).

Ewing and his co-workers first described single cell analysis by CE with electrochemical (EC) detection (Bergquist et al., 1997). Yeung's group analyzed smaller cell using CE in combination with LIF (Yeung, 1999). Zhang and Jin successfully detected nine amino acids quantitatively and qualitatively of single erythrocyte via CE-LIF with a new derivatization method (Zhang and Jin, 2003). These studies focused on two related techniques: one is to make efficient detection, the other is to minimize the dilution of the contents of a single cell during derivatization, so as to maintain favorable kinetics for the labeling reaction and to diluting the analytes already presented at trace levels.

The detecting methods of CE in amino acid analysis have been developed recently, including detections of UV, LIF, EC, mass spectrometry and miscellaneous (Poinsot et al., 2003). There are a few works concerning direct UV detection of amino acids. Although it was successfully tested for the separation and quantification of amino acids in physiological fluid, such as plasma and macrophage culture supernatant, it is difficult to detect the amino acids in single cell because the limits of this detection ranged from 1.93 μmol/L to 28.98 μmol/L (Zunic et al., 2002). While EC could be used to determine amino acids in a single cell, the limits of detection are only between 0.5 μmol/L and 28 μmol/L (Poinsot et al., 2003). Connecting MS to CE for analyzing amino acids of single cell was reviewed by Moini (2002). The reasons for the less use of MS-CE are that both MS is expensive and the detection is not sensitive due to the lower molecular weight of amino acid. As for miscellaneous routes of conductimetry and interfaced thermal lens microscope for CE, the former is suitable for resolution of cystine from matrix interferences present in the urine samples; the latter provides a 100-folds sensitivity over conventional absorbance detection, with the least detecting concentration of derivatized amino acids (about

24 nmol/L) (Poinsot et al., 2003). A final concentration of 0.15 nmol/L of LIF detection could reach 10^5 higher sensitivity than UV absorbance. The improvement of LIF detection involved the use of a new wavelength, which replaced the expensive lasers and a lower labeling concentration.

Derivatization is mainly used for enhancing the detection sensitivity of CE, especially in combination with LIF. Derivatization procedures are classified into pre-, on-, post-capillary derivatization and intracellular derivatization. It was used to derivatize amino acids in mammalian cells, with a dilution factor much more than ca. 100. On-column derivatization reported is effective for reducing the dilution, but the dilution factor was still high (ca. 100). The method of intracellular FITC-derivatization by electroporation made dilution factor reduced from ca. 100 to ca. 1.6 (Zhang and Jin, 2003).

These researches make us conclude that CE-LIF is a suitable approach for amino acid analysis of a single-cell. But most of the single-cell analyzed was conducted in human erythrocytes, lymphocyte mammalian cells and other animal cells. There were fewer researches on plant cells because of the hard cell wall and no success in plant protoplasts before we reported it recently (Chen et al., 2005). It is necessary to develop an efficient approach for amino acid analysis in single-cell level, so that we can monitor the relationship of its types and contents to plant physiology and development, as well as the nutrition of economical and food plants for human health. Common wheat (*Triticum aestivum* L. 2n = 42) is one of the most important crop species in the world, which can provide different kinds of food well made for human. Its quality is decided by the content and type of amino acid. In order to understand the rule of production and metabolism, we try to establish a method of detecting amino acid in common wheat. In this paper, we describe a pathway for checking the concentration of amino acids in both lysate and single embryonic cell of wheat under tissue culture.

Materials and methods

Materials

1.00×10^{-3} mol/L stock solutions of twenty standard amino acids (Shanghai Biochemical Reagents Co. China, chromatographic grade) were prepared with borax-NaOH buffer (pH10) respectively. A 2.0×10^{-4} mol/L FITC (FITC isomer I content 98%, Sigma, USA, chromatographic grade) solution was prepared with 1.25×10^{-2} mol/L borax/4.3×10^{-2} mol/L NaOH (pH 10). A 5% w/v SSA was prepared by dissolving in double distilled water. All reagents used in this experiment were analytical grade except for amino acids and FITC. All solutions were prepared with double distilled water.

Preparation and lysate of protoplasts from embryonic calli of common wheat

Common wheat (*Triticum aestivum*. cv. Jinan 177) embryonic cells (protoplasts) were derived from nodule structure on the surface of type II embryogenic calli of wheat (Guo et al., 1991). These protoplasts can be developed into somatic embryos and plants with a high frequency (Guo et al., 1991). The enzyme solution for isolation of the protoplasts contains 0.6 mol/L mannitol, 5 mmol/L $CaCl_2$, 1.5% cellulase Onozyka RS and 0.3% pectolyase Y-23, pH 5.8. The protoplasts were purified from the enzyme mixture with the same method as described by Guo et al. (1991). Resuspend the protoplasts in a osmotic buffer (0.6 mol/L mannitol, 5 mmol/L $CaCl_2$) to a density of about $4 \times 10^4/\mu$L. Then, spun the protoplasts solution (200 μL) at $60 \times g$ for 5 min, removed the supernatant fluid, and then resuspend the protoplasts with 1.5 mL 1.25×10^{-2} mol/L borax/1.25×10^{-2} mol/L NaOH (pH 9.4) . This produces the lysate of common wheat embryonic protoplasts.

Derivatization of standard amino acids and amino acids in lysate

1) Derivatization of standard amino acids was as follows: a stock solution of amino acids diluted to 1.00×10^{-4} mol/L with a 1.25×10^{-2} mol/L borax/4.3×10^{-2} mol/L NaOH (pH 10) was mixed with the borax/NaOH buffer (pH 10) containing 2.0×10^{-4} mol/L FITC in the same volume. Then the solution was diluted to 2.0×10^{-7} mol/L with the borax/NaOH buffer after 15 h of co-culturing in the dark.
2) Derivatization of amino acids in common wheat embryonic protoplasts lysate was conducted orderly: adding 400 μL of 5% w/v sulfosalicylic acid (SSA) to 1.5 mL lysate to precipitate the protein; spinning the mixture at $500 \times g$ for 20 min; mixing the supernatant liquid with 2.0×10^{-4} mol/L FITC in the same volume and then adjusting it to pH 10 with NaOH. After derivatization for 15 h at room temperature in the dark, the solution was diluted with the same buffer and then was determined.

Protoplast intracellular derivatization with electroporation

The electroporation equipment used here was in accordance with the previous work (Dong and Jin, 2001). 10 μL protoplast suspension was diluted to 0.1 mL with osmotic buffer (0.6 mol/L mannitol, 5 mmol/L $CaCl_2$) containing 2.0×10^{-4} mol/L FITC. Then the protoplasts in the suspension were electroporated according to Dong and Jin (2001). Since the protoplasts electroporated were brown, they could be monitored under the inverted microscope. The suspension was allowed to stand for more than 2 h at room temperature until the cells subsided to the bottom, and then the supernatant liquid was removed. Resuspend the protoplasts in the osmotic buffer and stand for other 2 h, with four to five times of repeat. After derivatization for 40 h in the dark at room temperature, the amino acids in embryonic protoplasts were determined.

Capillary electrophoresis separation of amino acids with LIF detection

A P/ACE MDQ capillary electrophoresis system equipped with an LIF detector (488 nm laser module) (Fullerton, CA, USA) was used to perform all separation and detection. Fluorescence was excited by an argon ion laser at 488 nm and was detected at 520 nm after passing through interference filter. The capillary with 25 μm ID was purchased from Yongnian Optical Conductive Fiber Plant (Yongnian China). The separation parameters were as follows: capillary 60.2 cm; effective length 50 cm; coolant tubing 42.8 cm; separation voltage 25 kV; coolant 25°C. Before each run, the capillaries were flushed for 3 min at 20 psi, with 0.1 mol/L NaOH, double distilled water, and separation buffer, respectively. After the electroosmotic flow reached a constant value, the derivatized standard

amino acid solutions or derivatized lysate was pressured into the capillary. Electropherograms were recorded and amino acids were determined after the separation voltage of 20 kV was applied.

Single protoplast analysis was in accordance with Zhang and Jin (2003). Finally, the separation voltage was applied and the electropherogram was recorded.

Results and discussion

Parameters for determination of amino acids

The electropherograms of FITC dissolved in 25×10^{-2} mol/L borax/1.25×10^{-2} mol/L NaOH-1.20×10^{-4} mol/L spermine (pH 9.45) and the standard solutions containing 20 amino acids derivatized by FITC are shown in Figs. 1 and 2 using a separation voltage of 25 kV. FITC formed one big peak and five small peaks of impurities in FITC. Nine amino acids Arg, Lys, Asn, Ser, Ala, Cys, Gly, Glu and Asp could be detected according to Fig. 2. However, other eleven ones, including Pro, Gln, Ile, Leu, His, Val, Met, Phe, Tyr, Asn, and Tyr, could not be identified, because their peaks have not been separated under the present conditions. Arg had the shortest migration time and Asp had the longest migration time among the nine separated well amino acids. The concentration limits of detections (CLODs) of every one of the nine separated well amino acids were determined with S/N as 3, respectively, and listed in Table 1. The linear relationship exists between the peak height, h, and the concentration, ranging from the CLODs to the highest concentration 1.0×10^{-6} mol/L used in this experiment, respectively (Table 1). It was also shown that the relative

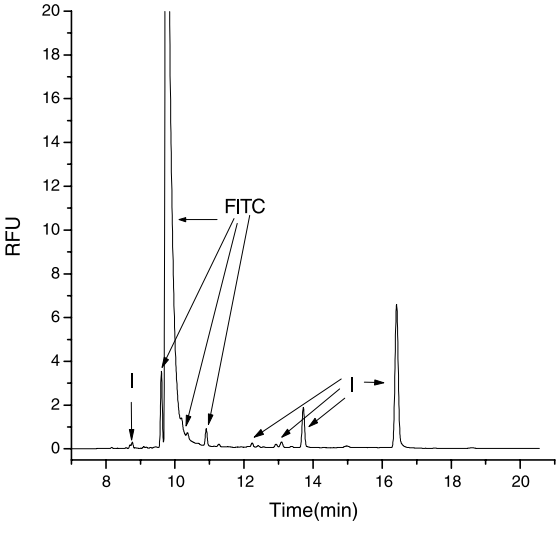

Fig. 1. Electropherogram of 8.0×10^{-6} mol/L FITC. *I*, Peaks of impurities in FITC; *RFU*, Relative fluorescence unit

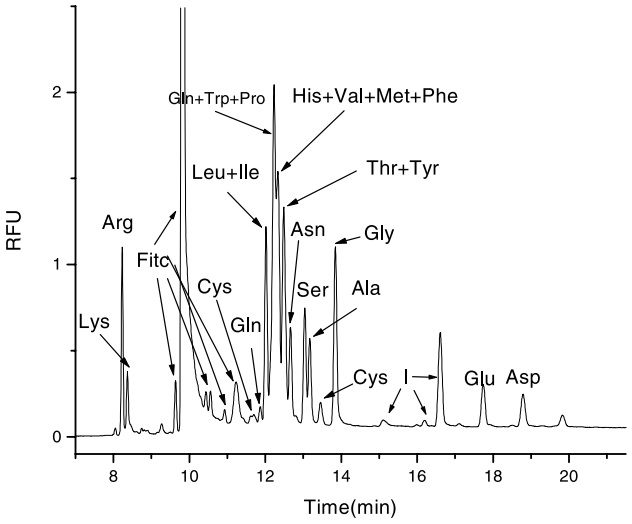

Fig. 2. Electropherogram of the standard solution containing 20 amino acids of 2.0×10^{-7} mol/L after derivatizing with FITC. Using 1.25×10^{-2} mol/L borax/1.25×10^{-2} mol/L NaOH 1.20×10^{-4} mol/L spermine (pH 9.45); injecting 4 psi for 5 s; selecting 25 kV separation voltage. *I*, Peaks of impurities in FITC

standard deviation of the method for a series of five injection of these amino acids of 2.0×10^{-7} mol/L are between 0.25–0.70% for t_m and 1.0–15% for h, respectively (Table 1). Comparing with previous work (Zhang and Jin, 2003; Zhang et al., 2005), we can find that the migration time and CLODs of every same amino acid, which were well separated, have a little difference. For example, the migration time of Arg in Zhang et al. (2005) was lower than that in this paper. So, we can conclude that new linear equation should be rebuilt when another concentration of amino acids was determined.

Determination of amino acids in common wheat embryonic protoplast lysate

By comparing the electropherograms of Fig. 1 with Fig. 2 and in combination with the relative migration times, the same 9 amino acids (Arg, Lys, Asn, Ser, Ala, Cys, Gly, Glu and Asp) can be found in embryonic protoplast lysate as well as in standard amino acids solution (Fig. 3). The mean concentrations of amino acids in the lysate obtained by the calibration curve for five determinations are listed in Table 2. Certain amounts of standard amino acids were added to the lysate and then the lysate was measured in order to prove the reliability of this method. From the detected concentration in the lysate with and without the standard amino acids, the recoveries are calculated and listed in Table 2 with their standard

Table 1. Relative parameters of nine amino acids after derivatized with FITC

Amino acids	Linear equation	Correlation coefficient	RSD_{t_m}(%)	RSD_h(%)	CLOD 10^{-10} mol/L
Arg	$Y = 3.0 \times 10^6 X + 0.0197$	0.9996	0.44	1.0	7.6
Lys	$Y = 8.5 \times 10^5 X + 0.0061$	0.9999	0.32	2.3	7.0
Asn	$Y = 1.0 \times 10^6 X + 0.0049$	0.9974	0.25	4.4	4.0
Ser	$Y = 2.0 \times 10^6 X + 0.0108$	0.9995	0.67	4.1	4.7
Ala	$Y = 2.0 \times 10^6 X + 0.011$	0.9995	0.70	8.5	5.1
Cys	$Y = 9.6 \times 10^5 X + 0.0193$	0.9988	0.69	9.8	5.8
Gly	$Y = 3.0 \times 10^6 X - 0.0038$	0.9993	0.69	4.9	16
Glu	$Y = 6.4 \times 10^5 X + 0.0119$	0.9987	0.46	7.6	5.0
Asp	$Y = 4.6 \times 10^5 X + 0.0093$	0.9989	0.46	15	9.7

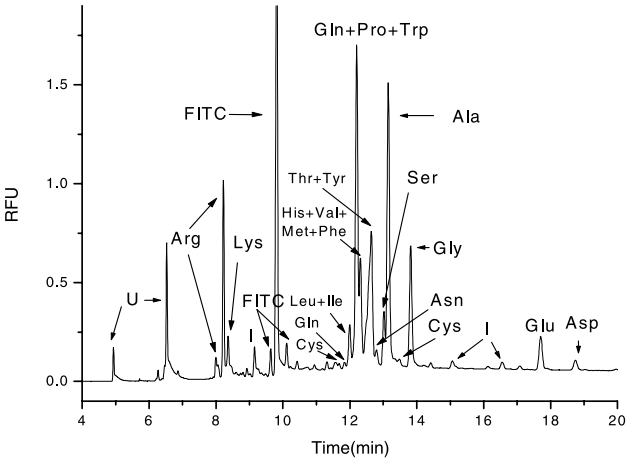

Fig. 3. Electropherogram of the amino acids after derivatized with FITC in lysate from embryonic cells. *U*, Unidentified peaks; *I*, Peaks of impurities in FITC

deviation ($n = 5$). The recoveries of the methods for the 9 amino acids are between 89% and 108%. Based on a protoplast concentration of 2.0×10^3 cells/μL in the lysate and a mean diameter (25 μm) of a single protoplast and the 240 times of detecting volume of lysate, the mean concentrations of amino acids in a single cell are calculated and listed in Table 2 with their standard deviation ($n = 5$). In comparison with the mean concentrations of hemolysate amino acids in one human erythrocyte (Zhang and Jin, 2003), most of the mean concentration of individual amino acid in common wheat embryonic protoplast is lower.

Analysis of amino acids in single protoplast

After a protoplast was injected and adsorbed on the wall of the capillary, 0.1 mol/L NaOH, as the cell lysis solution, was injected into the capillary for 2 s. The individual protoplast was lysed within 15 s. After the protoplast was lysed, a separation voltage of 25 kV was applied and the electropherogram of the protoplast was recorded (Fig. 4). Peak identification was carried out through electropherogram of the sample AAs in comparison with the standard AAs. Total ten amino acids, Arg, Lys, Trp, Thr, Asn, Ser, Ala, Cys, Gly and Glu, in an embryonic cell have been detected. However, other ten AAs have not been identified because their peaks could not be separated under the present conditions. The result shows that the external stan-

Table 2. Mean concentration of amino acids in the lysate, as well as in single embryonic cell

Amino acids	Mean concentration in the lysate ± SD (n = 5, 10^{-8} mol/L)	Recovery ± SD (n = 5) (%)	Mean concentration in single protoplast (10^{-5} mol/L)
Arg	34.0 ± 0.029	98.5 ± 3.8	8.30
Lys	28.6 ± 0.035	90.7 ± 2.6	6.98
Asn	19.5 ± 0.019	104.3 ± 3.2	4.76
Ser	18.3 ± 0.011	103.3 ± 1.5	4.64
Ala	74.5 ± 0.020	99.6 ± 0.6	18.18
Cys	11.0 ± 0.027	89.4 ± 2.6	2.68
Gly	35.5 ± 0.017	104.9 ± 7.8	8.66
Glu	37.0 ± 0.010	99.7 ± 6.5	9.02
Asp	24.9 ± 0.019	99.2 ± 3.5	6.08

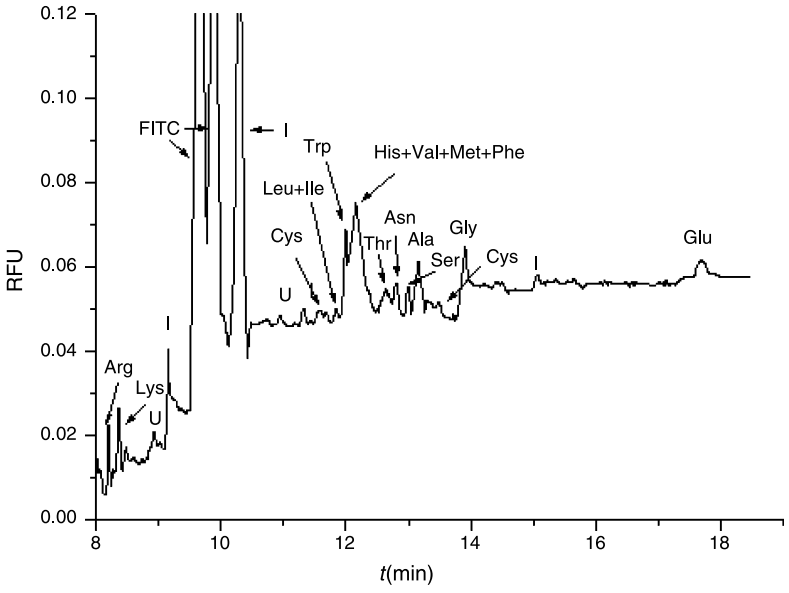

Fig. 4. Electropherogram of amino acids in one protoplast with intracellular FITC derivatization. The mean concentration and recover used the same as in Fig. 2. *U*, Unidentified peaks; *I*, Peaks of impurities in FITC

dardization can be successfully used for quantification of AAs in single cell.

We first described amino acids analysis in a common wheat embryonic protoplast by using capillary electrophoresis with LIF detection, in combination with tissue culture. In the previous work, vitamin C in pea and orange cells were determined using amperometer and direct UV detection (Poinsot et al., 2003).

Till now, there are only a few reports on the relationship of amino acids with the plant development. This literature provides a new and simple system for further study on the relation of embryo origin and development to amino acids through identify the different developing stages of common wheat embryonic cells.

Concluding remarks

In sum, a method to determine amino acids in individual cell of common wheat was established. In this method, protoplasts were acquired by digesting the common wheat cultural cells with enzyme solution. PBS buffer was replaced by osmotic buffer in protoplasts derivatization in order to keep osmotic balance between out and in the protoplast. At the same time, the method for derivatization of protoplast could also be used in other high plant species to measure the content of amino acids in single cell.

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