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Characterization of developmental autolysis in myxobacterial fruiting body morphogenesis with profiling of amino acids using capillary electrophoresis method

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Summary. Capillary electrophoresis equipped with Laser-induced fluorescence (CE-LIF), combining with micro-culture technique was employed to determine extracellular amino acids in single myxobacterial fruiting body morphogenesis. The result showed that in the early aggregation stage, there was a remarkable increase of extracellular amino acids, which was produced by developmentally induced autolysis. The amino acids were then consumed by the vegetative cells in aggregation stage. In the following developmental events, the extracellular amino acids were kept at low level, which indicated that in the stages of fruiting body formation and myxospore development, there was no further cell autolysis. Using this novel method may provide detailed insight into the mechanisms of the developmental phenomena.

Keywords: Capillary electrophoresis – Laser-induced fluorescence detection – Myxobacterium – Morphogenesis – Autolysis

Introduction

Myxobacteria are gram-negative, usually soil-dwelling bacteria that feed on other organisms or macromolecular organic matters (Reichenbach, 1993; Dawid, 2000). The myxobacteria carry out cooperative behavior throughout their life cycle and this complex social behavior, especially the morphogenesis of fruiting bodies, provides an ideal model for the study of cell-to-cell communication and cooperation in prokaryotes (Shimkets, 1990; Dworkin, 1996; Kaiser, 2003). When starved, myxobacterial cells, 5–7 μm–0.5 μm in size, migrate on surface developing fruiting bodies. During the morphogenetic progress, high density cells aggregate on solid surface to build multicellular resting structure-fruiting bodies, a developmental culmination of a series of events set in motion by the changing nutritional and physical environment. Inside fruiting bodies, most of the vegetative cells then differentiate into adversity-resistant myxospores. Morphogenesis is regulated by several signals (Dworkin, 1996; Skimkets, 1999; Jelsbak et al., 2000; Kaiser, 2004). For instance, the diffusible, quorum sensing A-signal; a mixture of amino acids and peptides generated by a mixture of proteases initiates the vegetative cells to aggregate; C-signal, short-chain alcohol dehydrogenase is a cell surface bound protein that coordinates the motion of individual cells by cell-contact.

As a number of genetic and microbiological studies indicate, development of a myxobacterial fruiting body is induced by environmental factors that inhibit growth. During fruiting body formation in *M. xanthus*, Wireman and Dworkin observed that approximately 60–80% of the original vegetative population autolysed and the surviving cells completed morphogenesis (Wireman and Dworkin, 1975, 1977). Shimkets proposed several possibilities to explain the function of developmental autolysis (Skimkets, 1990) and Kaiser and Kroos indicated that a mixture of amino acids or small peptides might play an important role in morphogenesis (Kaiser and Kroos, 1993). However, there is no report describing the developmental changes of amino acids or small peptides, which may be important for elucidation of morphogenesis mechanisms. In this paper, we tried to develop a method to assay the chemical changes occurring during morphogenesis of a single fruiting body.

Capillary electrophoresis (CE) has been used for separation and quantification of chemicals, including amino acids, in complicated bio-systems because of its inherent features suitable for analysis of a very small amount of

samples, high separation speed and efficiency and biocompatible operation environments (Yeung, 1999). A laser-induced fluorescence (LIF) detector, equipped on CE offers high sensitivity for analytes. Many publications have dealt with the separation and detection of amino acids using CE-LIF (Smith, 1999). A technique for the determination of 20 amino acids in CE-LIF was reported with a concentration limit of detection (CLOD) of 2.0–9.4 $\times$ 10^{-10} mol/L (Zhang and Jin, 2004). In this work, we applied CE-LIF to profile and quantify the extracellular amino acids in one single myxobacterial fruiting body for characterization of developmental autolysis in myxobacterial fruiting body morphogenesis.

Materials and methods

Materials

All standard amino acids (chromatographic grade) were purchased from Shanghai Biochemical Reagent Co. (Shanghai, China). 1×10^{-4} mol/L sock solution of amino acids was prepared by dissolving appropriate amounts of amino acids in borax-NaOH buffer (pH 10). FITC isomer I (content 98%, HPLC) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A 2×10^{-4} mol/L FITC solution was prepared with the same buffer. All reagents were of analytical grade except for amino acids and FITC. All solutions were prepared with double-distilled water and stored at 4°C.

Organism and cultivation

The myxobacterial strain, *Myxococcus fulvus* HW-1 (ATCC BAA-855) was isolated from a seawater sample (Li et al., 2002). The strain was routinely cultivated on VY/2 agar containing 1.5% agar, 0.5% yeast, 0.05% $CaCl_2$, 0.05% $MgSO_4$ and 0.5 μg/ml vitamin B12, pH 7.2 (Reichenbach and Dworkin, 1992). Water-agar containing 0.05% $CaCl_2$ and 0.05% $MgCl_2$ was used as developmental medium for fruiting body formation, which was performed in 96-well plate to control the cell number and culture volume for each assay. All the cultures were incubated at 30°C.

To quantify the cell numbers involving in one single fruiting body formation, the vegetative cells from VY/2 in exponential stage were harvested and gently homogenized with small glass beads. The cell suspension was centrifuged at 5,000 rpm for 5 min. The cell mass was then washed with distilled water and centrifuged three times to remove any attached particles and extracellular metabolites. After counting and adjusting to ca.10^8 cells per milliliter, 10 μl of the cell suspension was inoculated into 96-well culture plate, in which containing 200 μl developmental medium. In this condition, the cells were determined to be usually able to form a few numbers of fruiting bodies per well. The number of fruiting bodies in wells was counted and the average was used as the fruiting ability from the cells in each well.

Extracellular amino acid extraction

The whole cell mass on the agar in one well was harvested at intervals of 2 h. Five replicates were performed. The cells were transferred into a micro-centrifuge tube containing 20 μL distilled water, vibrated to disperse the cells and extract the extracellular dissolvable contents for 4 h at room temperature. The mixture was centrifuged and the supernatant was transferred into a new micro-centrifuge tube to dry at room temperature. The remainders were resolved in 10 μL 1.25×10^2 mol/L borax-NaOH buffer, pH 10. The solution was stored at 4°C. Meanwhile, the agar in the wells was also harvested, extracted with distilled water, lyophilized and resolved in the same buffer.

Pre-column derivatization of standard amino acids and sample

A stock solution of standard amino acids or sample was mixed with the borax-NaOH buffer containing FITC with the same volume. After derivatization for 15 h in the dark at room temperature, the solution was diluted with the same buffer and then was determined.

Capillary electrophoresis separation of amino acids with LIF detection

All separations and detection were performed on a Beckman P/ACE MDQ commercial capillary electrophoresis system (Fullerton, CA, USA) with a LIF detector (488 nm Laser Module, Beckman Coulter). Fluorescence was excited by argon in laser at 488 nm (3 mW) and was detected at 520 nm after passing through interference filter. The capillaries with 50 μm ID were supplied from Yongnian Optical Conductive Fiber Plant (Yongnian, China). All separations were performed on an untreated capillary with total length of 60.2 cm and effective length of 50 cm. The length of the coolant tubing was 42.8 cm. The sample temperature of 25°C and coolant temperature of 25°C were maintained throughout. Before each run, the capillary was rinsed with 0.2 mol/L NaOH, double-distilled water, and separation buffer for 3 min, respectively, using 20 psi. The derivatized standard solutions or the derivatized sample was introduced into the capillary with pressure. The separation voltage of 20 kV (normal polarity) was applied, all data were collected and the electropherogram was recorded.

Results and discussion

The developmental events during fruiting body formation by the myxobacterium were characterized by Wireman and Dworkin as several sequential stages: growth – aggregation – formation of raised mounds of cells – autolysis – myxospore development (Wireman and Dworkin, 1977). Wireman and Dworkin (1975 and 1977) claimed that 60 to 80% of the cell population underwent autolysis during development. They determined the phenomenon using three methods: (i) determination of colony-forming units, (ii) microscopic examination of cell numbers, and (iii) detection of [*methyl*-^{3}H] thymidine-tagged DNA throughout the course of development. They suggested that the vegetative cell autolysis might play an important role in the developmental events, *e.g.* under condition of nutrient deprivation, individual cells needed either sufficient endogenous reserves for myxospore induction (*e.g.* capsule synthesis) or an exogenous energy source; a fraction of the population provided, by autolysis, some essential nutrients such as amino acids for the survival of the remainder of the population. But O'Connor and Zusman found little convincing evidence for massive cell autolysis using the same methods mentioned above using scanning electron microscopy (O'Connor and Zusman, 1988). Rosenberg

et al. further argued that there was cell-density-dependent lysis using immobilized cells (Gelvan et al., 1987). Whether there is developmental autolysis and whether the autolysis (if existing) plays an important role in the morphogenesis might have to be answered from the analysis of the extracellular chemical changes during the developmental process of one single fruiting body. However, there is hence no report on the item of autolysis for over 15 years because of difficulties of micromanipulation and quantification method. Amino acids are the main dissolvable content in cytoplasm. If massive autolysis occurs in the developmental events, it must result in obvious changes of extracellular amino acids. In our experiments, a CE approach combined with microculture, was introduced to quantitatively measure the content of extracellular amino acids during one single fruiting body morphogenesis to characterize the autolysis events.

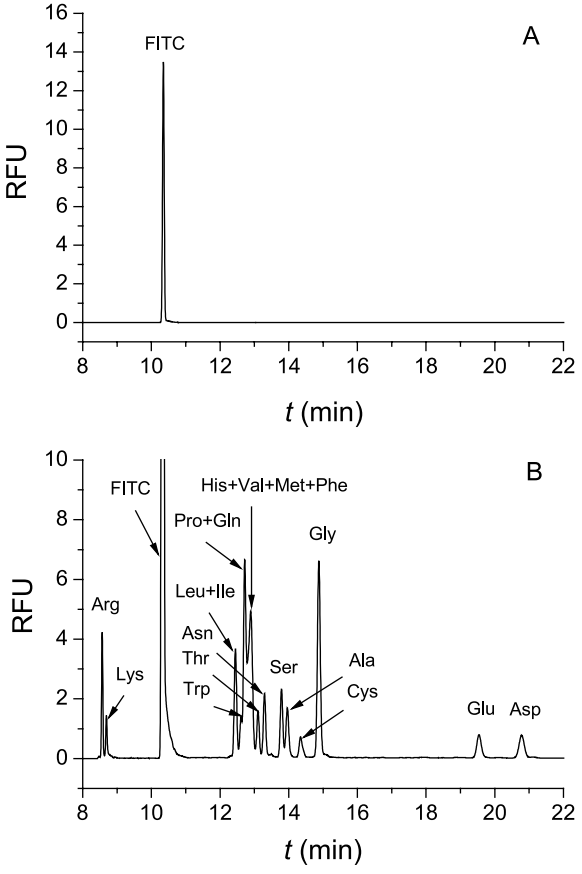

Fig. 1. Electropherograms of **A** 10^{-6} mol/L FITC and **B** the 19 standard amino acids. CE running buffer, 2.0×10^{-2} mol/L borax-8.0×10^{-2} mol/L NaOH (pH 9.4); injection, 0.5 psi for 5 s; separation voltage, 20 kV (normal polarity). *RFU*, relative fluorescence unit

FITC in 2.0×10^{-2} mol/L borax – 8.0×10^{-2} mol/L NaOH (pH 9.4) and the mixture containing 19 amino acids derivatized by FITC with a separation voltage of 20 kV is shown in Fig. 1. FITC formed only one peak with elution time of ca. 10.3 min (curve A) and 10 amino acids (Arg, Lys, Thr, Asn, Ser, Ala, Cys, Gly, Glu, Asp) were well separated in the presence of other amino acids according to this electropherogram (curve B). Leu and Ile were eluted at the same time, Gln overlapped Pro, and His, Val, Met and Phe overlapped in one peak, the peak of Trp is truncated by the peak of Pro + Gln. Arg had the shortest migration time (t_m) and Asp had the longest t_m. The CLOD of every one of the 10 amino acids, which were well separated, was determined with S/N as 3, respectively, and listed in Table 1. It was found that the CLODs ranged from 8.8×10^{-10} mol/L for Gly to 2.9×10^{-9} mol/L for Arg. The linear relationship exists between peak height detected and concentration, ranging from the CLOD to the highest concentration 1×10^{-5} mol/L used in this experiment, respectively.

Figure 2 summarizes the stages of fruiting body formation in *Myxococcus fulvus HW-1* on an argar surface under

Table 1. Analysis data of 10 standard amino acids

Amino acid	Linear range (mol/L)	Slope (RFU/μmol·L^{-1})	*y*-intercept (RFU)	Correlation coefficient	CLOD (10^{-9} mol/L)
Arg	2.9×10^{-9}–1×10^{-5}	8.54	4.21×10^{-3}	0.9975	2.9
Lys	2.3×10^{-9}–1×10^{-5}	2.90	1.23×10^{-3}	0.9952	2.3
Thr	2.3×10^{-9}–1×10^{-5}	5.16	2.79×10^{-3}	0.9990	2.3
Asn	1.1×10^{-9}–1×10^{-5}	4.93	7.30×10^{-4}	0.9955	1.1
Ser	2.1×10^{-9}–1×10^{-5}	5.71	2.08×10^{-3}	0.9989	2.1
Ala	1.9×10^{-9}–1×10^{-5}	3.55	3.70×10^{-4}	0.9949	1.9
Cys	2.0×10^{-9}–1×10^{-5}	2.34	3.20×10^{-4}	0.9960	2.0
Gly	8.8×10^{-10}–1×10^{-5}	13.3	2.64×10^{-3}	0.9983	0.88
Glu	1.0×10^{-9}–1×10^{-5}	2.12	4.90×10^{-4}	0.9962	1.0
Asp	1.2×10^{-9}–1×10^{-5}	2.03	4.30×10^{-4}	0.9995	1.2

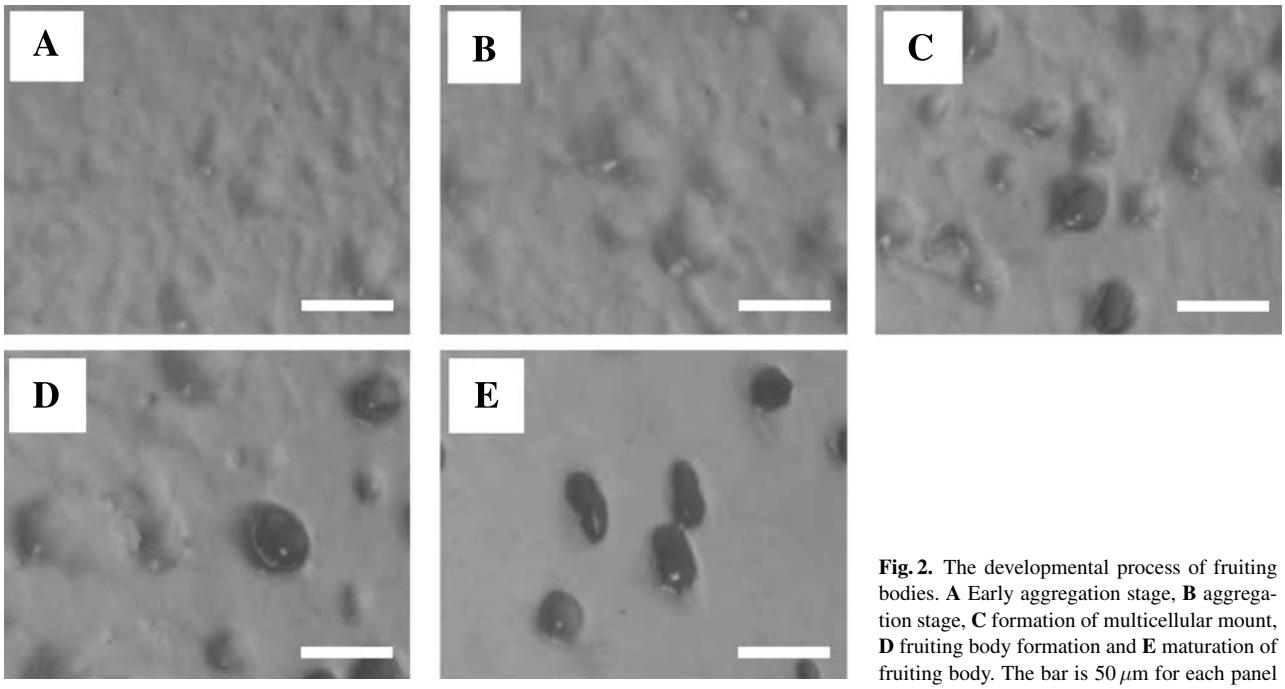

Fig. 2. The developmental process of fruiting bodies. **A** Early aggregation stage, **B** aggregation stage, **C** formation of multicellular mount, **D** fruiting body formation and **E** maturation of fruiting body. The bar is 50 μm for each panel

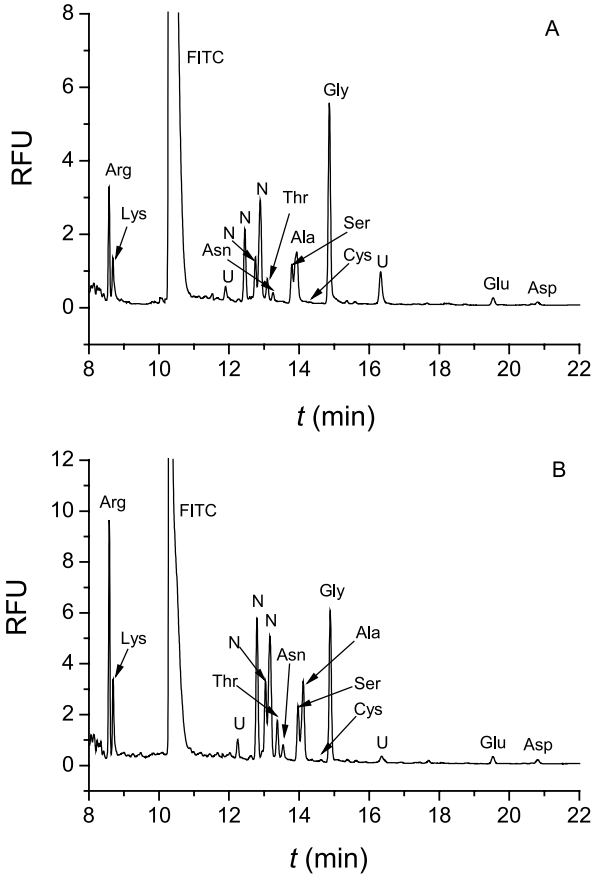

Fig. 3. Electropherograms of amino acids in two samples. Condition as in Fig. 1. Peaks unidentified are marked as U

the microscope. Fig. 2A and B show stages of vegetative cell aggregation. The earliest stage is slightly elevated (Fig. 2A) and the aggregation centers continue to develop, the central portion differentiates, resulting in a fried-egg appearance: formation of multicellular mount and fruiting body formation (Fig. 2C and D). The central portion of the fried egg finally develops into fruiting body with discernible colour.

The extracellular amino acid contents in different stages (A–E, shown in Fig. 2) were determined according to the observation under the microscope. Figure 3 shows two typical electropherograms of amino acids at stage A (curve A) and at stage B (curve B). For the two electropherograms, the highest peak corresponds to FITC and ten different amino acids were marked, respectively. Identification of the amino acids was conducted by comparison with the electropherogram of Fig. 1B and spiking the corresponding standard amino acids. It is difficult to identify the peak noted as N, because several amino acids have the same eluting time. In addition, there are several unknown peaks noted as U appearing in the electropherograms.

The reproducible peak currents together with the large linear range for standard amino acids make it suitable to utilize external standardization for quantification of the well-separated amino acids in the samples. The contents of the amino acids are proportional to the height of the peaks. The content variation of the amino acids in

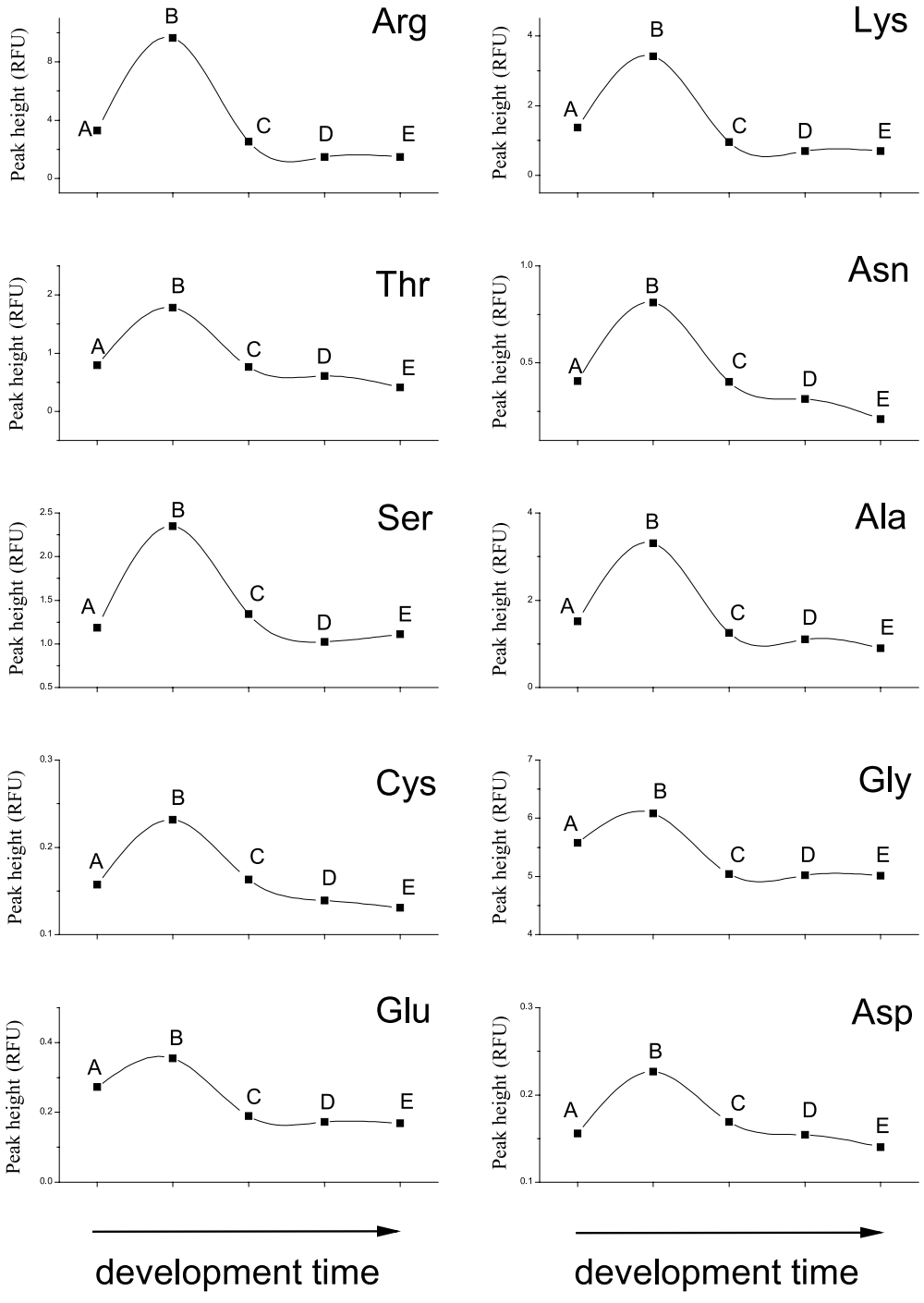

Fig. 4. Curves of amino acid content variation in raised mounds and fruiting bodies. Condition as in Fig. 1. A–E indicates the developmental stages as in Fig. 2

different stages was summarized in Fig. 4, the points represented the average of the amino acids detected for three runs (The peak height is proportional to the content of the extracellular amino acid and the letters of the points correspond to the ones in Fig. 2). It was found that the amino acids were at low level in stage A, rose from point A to B, reached the maximum (B point) at stage B and declined from point B to C, and the values of point C, D and E were almost at the same level. In order to avoid the error result of the amino acid determination produced by selecting sample, the content of amino acids extracted from agar were also determined and the result was

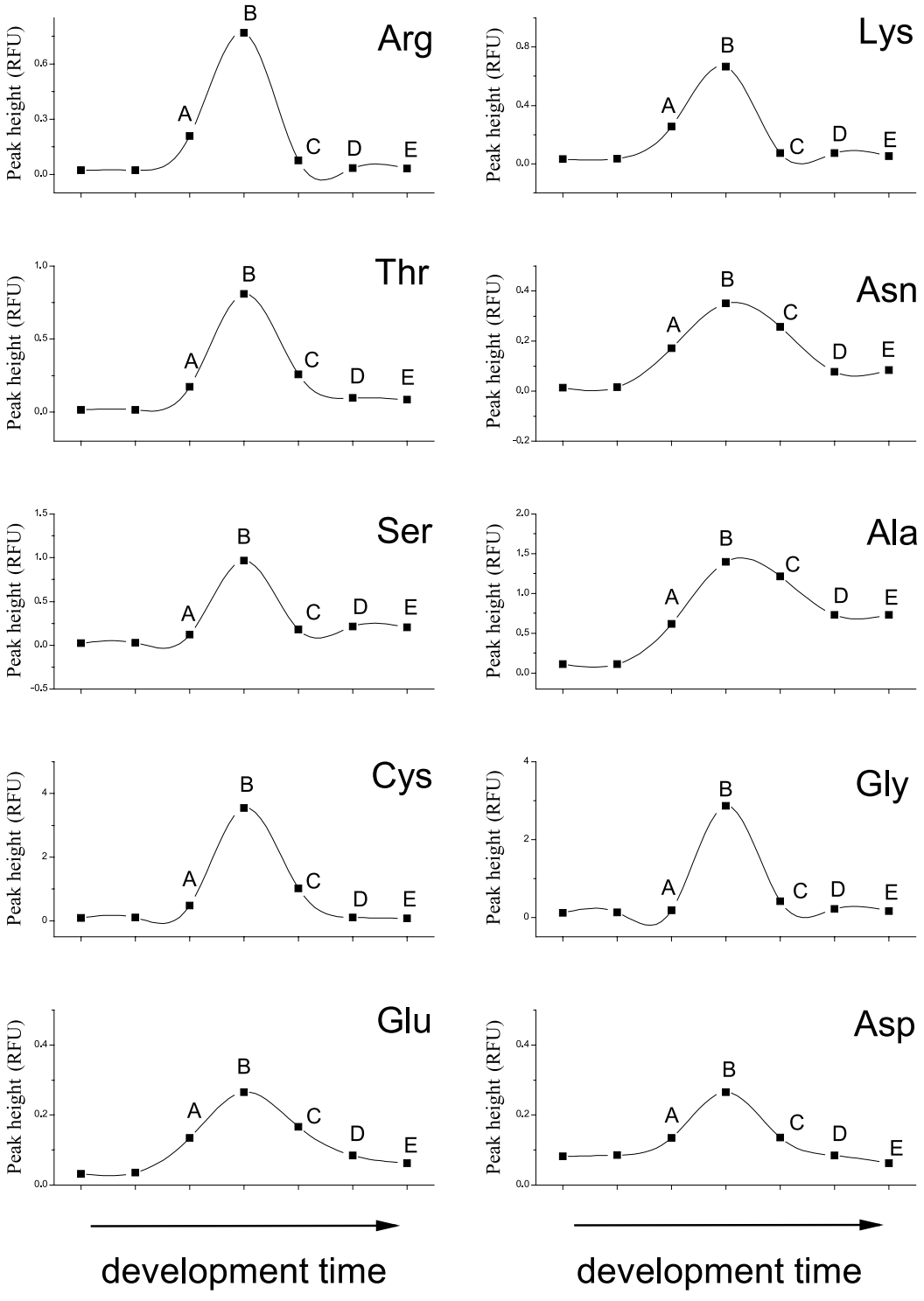

Fig. 5. Curves of amino acid content variation in culture medium. Condition as in Fig. 1. A–E indicates the developmental stages as in Fig. 2

summarized and shown in Fig. 5, which showed a similar curve as in Fig. 4.

The curves in Fig. 4 showed that autolysis appeared during stages A and B, which represented the aggregation stage of vegetative cells induced by nutrient starvation. After that, the surviving cells developed into fruiting bodies and myxospores. From the curves, it is clear that fruiting body formation consumes nutrients, which were

provided by autolysis of the vegetative cells. The consumption mainly appeared in the pre-stage of fruiting body formation. After the cell mounts formed, nearly no nutrients were needed by the cells. CE with LIF detection exhibits good advantages over the method reported (Dworkin, 1996; Kaiser, 2003; Wireman and Dworkin, 1977) for characterization of developmentally induced autolysis of myxobacterium because of extremely small sample size, high separation speed and efficiency and high sensitivity. Using this novel method, CE with LIF detection, may provide detailed insight into the mechanisms of such important developmental phenomena.

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References

Dawid W (2000) Biology and global distribution of myxobacteria in soils. FEMS Microbiol Rev 24: 403–427

Dworkin M (1996) Recent advances in the social and developmental biology of the myxobacteria. Microbiol Rev 60: 70–102

Gelvan I, Varon M, Rosenberg E (1987) Cell-density-dependent killing of *Myxococcus xanthus* by autocide AMV. J Bacteriol 170: 844–848

Jelsbak L, Søgaard-Andersen L (2000) Pattern formation: fruiting body morphogenesis in *Myxococcus xanthus*. Curr Opin Microbiol 3: 637–642

Kaiser D (2003) Coupling cell movement to multicellular development in myxobacteria. Nat Rev Microbiol 1: 45–54

Kaiser D (2004) Signaling in myxobacteria. Annu Rev Microbiol 58: 75–98

Kaiser D, Kroos L (1993) Intercellular signaling. In: Dworkin M, Kaiser D (eds) Myxobacteria II. Am Soc Microbiol, Washington, DC, pp 257–283

Li Y, Hu W, Zhang Y, Qiu Z, Zhang Y, Wu B (2002) A simple method to isolate salt-tolerant myxobacteria from marine samples. J Microbiol Meth 50: 205–209

O'Connor KA, Zusman DR (1988) Reexamination of the role of autolysis in the development of *Myxococcus xanthus*. J Bacteriol 170: 4103–4112

Reichenbach H (1993) Biology of the myxobacteria: ecology and taxonomy. In: Dworkin M, Kaiser D (eds) Myxobacteria II. Am Soc Microbiol, Washington, DC, pp 13–62

Reichenbach H, Dworkin M (1992) The myxobacteria. In: Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes, 2nd edn. Springer, Berlin Heidelberg New York Tokyo, pp 3416–3487

Shimkets LJ (1990) Social and developmental biology of the myxobacteria. Microbiol Rev 54: 473–501

Shimkets LJ (1999) Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. Annu Rev Microbiol 53: 525–549

Smith JT (1999) Recent advancements in amino acid analysis using capillary electrophoresis. Electrophoresis 20: 3078–3083

Wireman JW, Dworkin M (1975) Morphogenesis and developmental interactions in mycobacteria. Science 189: 516–523

Wireman JW, Dworkin M (1977) Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. J Bacteriol 129: 796–802

Yeung ES (1999) Study of single cells by using capillary electrophoresis and native fluorescence detection. J Chromatotgr A 830: 243–262

Zhang H, Jin W (2004) Analysis of amino acids in individual human erythrocytes by capillary electrophoresis with electroporation for intracellular derivatization and laser induced fluorescence detection. Electrophoresis 25: 480–486

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