생명과학
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Enoylpyruvate Transferase Isozymes in *Bacillus megaterium*

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1. Abstract

UDP-GlcNAc is metabolized to form vegetative cell wall, cortical peptidoglycans, and outermost layer consisting of galactosamine-6-phosphate polysaccharide in life cycle of *Bacillus megaterium*. To obtain a better understanding of the UDP-GlcNAc regulation, we examined the activity of the common first enzyme for the synthesis of nucleotide precursors of peptidoglycans, enoylpyruvate transferase by newly developed method. Both the specific and the total activity decreased after the end of exponential growth followed by an increase from t_5 but decreased again parallel to the appearance of the activity of UDP-GlcNAc-4-epimerase. Antibody specificity to anti-transferase IgG and the elution profile on DEAE-Sepharose revealed that *B. megaterium* has at least two enoylpyruvate transferase isozymes, and UDP-GlcNAc was metabolized to vegetative cell wall and cortical peptidoglycan by each isozme in exponential growth and in sporulation, respectively in life cycle.

Introduction

Bacillus megaterium has been known to metabolize UDP-GlcNAc to form different kinds of structures, namely, vegetative cell wall, cortex and outermost layer(OL) which Gerhart's group calls exosporium[10] in life cycle. Pitel and Gilvary[13] reported that upon the initiation of sporulation, vegetative cell mucopeptide synthesis ceased and three hours after the end of vegetative cell growth, spore-specific mucopeptide synthesis began and Tipper and coworker[4,5,16] investigated the variation in the activity of enzymes involved in synthesizing UDP-MurNAc-pentapeptide precursors of the cortex peptidoglycan in *B. sphaericus*. About the first enzyme for the synthesis of spore-specific galactosamine-6-phosphate polysaccharide of OL, UDP-GlcNAc-4-epimerase, we have demonstrated that an increase in the activity of this enzyme is parallel to the induction of enzyme synthesis by immunoassay.[11] But, nothing is known about enoylpyruvate transferase, a common first enzyme for the synthesis of nucleotide precursors of both vegetative cell wall and cortical peptidoglycans. Moreover, it was not still elucidated whether the activity of UDP-GlcNAc-4-epimerase was co-regulated with that of enoylpyruvate transferase later during sporulation. To gain a better understanding of the regulation of the metabolism of UDP-GlcNAc, we started to examine the activity of the transferase and characterized the enzyme. And here we have reported that *B. megaterium* has at least two enoylpyruvate transferase isozymes and UDP-GlcNAc was metab-

olized to vegetative cell wall and cortical peptidoglycans by the different isozymes in exponential growth and in sporulation, respectively in life cycle.

Materials and Methods

Bacterial strain and growth condition.

B. megaterium ATCC 12872 was used. Cells of *B. megaterium* were grown and sporulated in supplemented nutrient broth(SNB) medium at 30 ℃ as previously described.[11, 15] Phase-bright spores reach maximum at t_7(tn indicated n hr after the end of exponential growth) and this stage was used as an internal marker for the timing of developmental events.

Assay for enoylpyruvate transferase.

The reaction mixture consisting of 1.0 mM UDP-GlcNAc, 1.0 mM PEP, 0.5 mM DTT, 12.5 mM KF to inhibit the enolase reaction and the crude enzyme in 0.5 ml of 50mM Tris-HCI (pH7.5) was incubated at 37℃ for 30 min and the reaction was terminated at 100℃ for 3 min.

After precipitation of denatured protein by centrifugation for 5 min at 12,000 × g, a sample of supernatant was analyzed by HPLC. The control contained all components of the reaction mixture except PEP. Protein concentration was determined using the Bio-Rad Protein Assay Kit with bovine serum as a standard. UDP-GlcNAc-4-epimerase was assayed as previously described.[11] The HPLC was carried out with Shim-pack CLC-ODS (6.0mm × 15 cm, Shimazu) and the elution buffer used was 50 mM ammonium formate (pH 3.9). Flow rate 1.0ml/min and column temperature 20℃ were employed. UDP-GlcNAc-enoylpyruvate was detected by U.V. absorbance at 262 nm and its concentration was determined by comparison with a standard curve of UDP-GlcNAc.[11]

Preparation of UDP-GlcNAc-enoylpyruvate.

UDP-GlcNAc-enoylpyruvate was enzymatically prepared by the method of Gunetileke and Anwar[7] with slight modification. In summary, a reaction mixture containing UDP-GlcNAc, 40μmoles ; PEP, 80 μmoles ; DTT, 16 μmoles ; 400 μm moles ; and crude protein fraction, which was prepared by bringing the cell extract of 2 liter of the exponential growing culture of *B. megaterium* to 80% saturation with $(NH_4)_2SO_4$ and dissolving in 32 ml of 50 mM Tris-HCI buffer (pH 7.5), was incubated at 37℃ for 24 hrs. Deproteinized sample by ultrafiltration was chromatographed on a DEAE-Sephadex A-25 (1 × 17 cm)column by stepwise gradient elution with 0, 0.1, 0.2 and 0.25 M NaCl and the elutants with 0.2 M NaCl were chromatographed on a Sephadex G-50 (1.5 × 50 cm) column with deionized water. The 13 C-NMRK spectrum of purified UDP-GlcNAc enoylpyruvate was recorded at 23℃ in D^{20} solution using dioxane as an internal standard on a JEOL GX-400 FT-NMR spectrometer operation 100 MHz.

Purification of enoylpyruvate transferase in sporulation cells.

The following purification procedures were carried out ; all operations were performed at 0~4℃ unless otherwise stated. First, crude enzyme obtained from the sporulating cells at t_7 was brought to 70% saturation with $(NH_4)_2SO_4$ and the precipitate was removed by centrifugation and dissolved in 20 mM Tris-HCl buffer(pH 7.5) containing 4.0 mM DTT and 1.0mM EDTA (buffer A). Second, the solution was chromatographed on a Sephacryl

S-300 column(1.6 × 100 cm) with buffer A at a flow rate of 0.2ml/min. Under these conditions, the transferase-containing fractions were eluted at 1.7 times the void volume. Third, these fractions were loaded on a DEAE-Sepharose CL-6B column (1.0 × 10 cm) equilibrated with buffer A and chromatographed with a linear gradient of 0-0.3 M NaCl in buffer A at a flow rate of 0.15ml/min. The transferase activity peak was observed at approximately 0.1 M NaCl. Finally, the transferase were separated on a hydroxyapatite column(1.0 × 5.0 cm) previously equilibrated with 20 mM Tris-HCL buffer (pH 7.5) containing 4.0 mM DTT (buffer B). A linear gradient of 0-0.5 mM $MgSO_4$ in buffer B was applied at a flow rate of 0.12 ml/min and the transferase activity peak was observed at approximately 0.1 M $MgSO_4$.

Preparation of the antiserum of enoylpyruvate transferase.

Antiserum to enoylpyruvate transferase prepared by injecting of a rabbit intra muscularly five times with 20-50 μg of the purified enzyme protein in Freund's complete adjuvant once two weeks. IgG was prepared by fractionation of the serum with 50% $(NH_4)_2SO_4$, followed by passage through a column of DEAE-cellulose.[9]

SDS-polyacrylamide gel electrophoresis(SDS-PAGE) and immunoblot analysis.

SDS-PAGE was performed on 1 mm thick vertical slab gels of 12.5% polyacrylamide at a constant current of 30 mA at room temperature. Gels were stainned with Coomassie brilliant blue R-250. Immunoblot analysis was carried out by the method previously described.[9]

Preparation of cell extracts.

Growing or sporulating cells were harvested from 1 liter of the culture, washed with 0.9% NaCl, and suspended in 30-50 ml of 75 mM Tris-HCl buffer (pH 7.5) containing 4.0 Mm DTT and 1.0 mM EDTA. After disruption of the cells by sonication, the suspension was centrifuged at 15,000 × g for 30 min at 4℃. The supernatant was used for the source of crude enzymes.

Chemicals.

1) UDP-GlcNAc, PEP, (DTT, KF),[2] resign for chromatography and[3] 2nd-antibody. All reagents employed in this study were of analytical grade, unless otherwise stated.

Results

Enoylpyruvate transferase assay.

The new assay method for enoylpyruvate was developed, in which the rate of production of UDP-GlcNAc-enoylpyruvate is measured by HPLC. Purified UDP-GlcNAc-enoylpyruvate was a single peak by HPLC and two new peaks, COOH : 175.2 ppm, $CH^2=C$: 156.5 ppm were observed in 13 C-NMR spectrum in comparison with that of UDP-GlcNAc (data not shown). Optimal reaction conditions were examined for crude enzymes in exponentially growing cells. The optimal pH range for transferase activity was between 7.5 and 8.5 and Km for PEP, UDP-GlcNAc were estimated to be 0.07 mM and 0.24 mM, repectively. The rate of UDP-GlcNAc-enoylpyruvate formation increased linearly with increasing enzyme concentration from 0 to 10.6 munits/ml reaction mixture (Fig. 1).

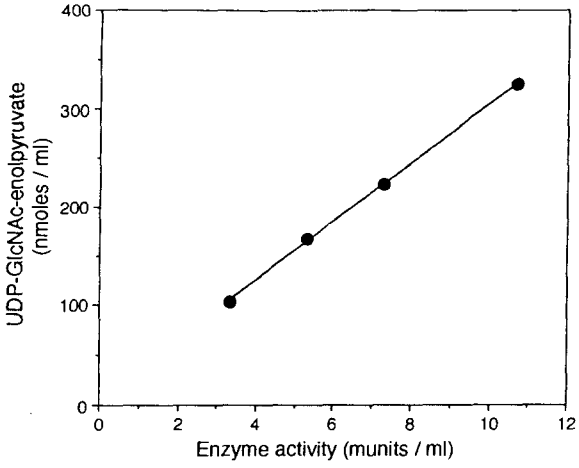


Fig. 1. Relation between enzyme activity and formation of UDP-GlcNAc-enoylpyruvate. Crude enzyme at sporulation phase was used for the measurement of enzyme activity. Enzyme activity was assayed as described in the materials and methods.

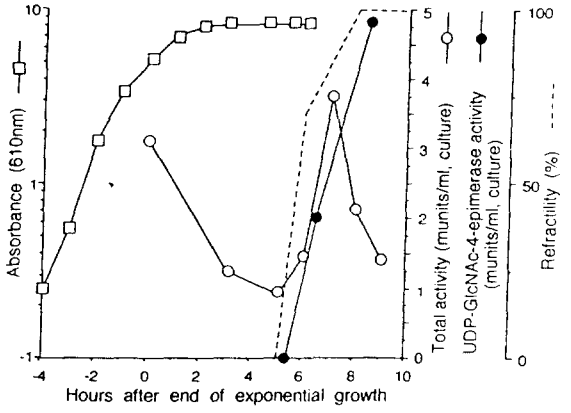


Fig. 2. Changes in enoylpyruvate transferase and UDP-GlcNAc-4-epimerase activity during life cycle. Enoylpyruvate transferase was assayed as described in materials and method. Open circles(○) represent total activity for enoylpyruvate transferase and solid circles (●) for total activity of UDP-GlcNAc-4-epimerase. Growth curve was measured by optical density (O.D.) at 610 nm (□). Forespores was monitored by phase contrast microscope, which was shown with dotted line(---).

Variation in the enoylpyruvate transferase activity in life cycle.

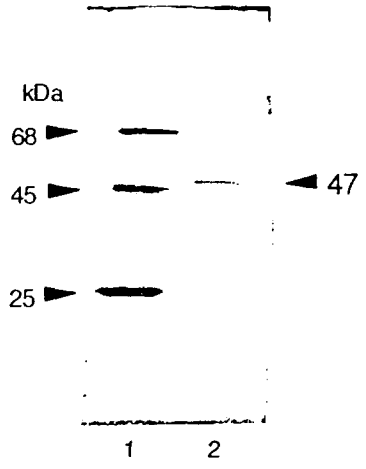
Both the specific and the total activities of transferase decreased after the end of exponential growth followed by an increased from about t_5, but decreased again parallel to the appearance of the activity of UDP-GlcNAc 4-epimerase (Fig. 2). The appearance of refractile forespores occurred at the same time as the increase of the transferase activity.

Purification of enoylpyruvate transferse in sporulation.

To understand the regulation mechanism of the transferase activity, it is important to elucidate whether the transferase in exponential growth is same or not as that in sporulation. First, the transferase in sporulation was purified from the crude enzyme in the sporulating cells at t_7 as described in materials and methods. An inactivation of the transferase was little by Sephacryl S-300 and DEAE-Sepharose CL-6B chromatography, but about 40~50% decrease of the activity was observed by ultrafiltration or dialysis, especially the enzyme was remarkably inactivated by hydroxyaptite chromatography. The final enzyme preparation after hydroxyapatite chromatography gave a single band in 12.5% SDS-PAGE at the molecular weight of about 47 kDa (Fig. 3).

Immunoblot analysis for cell extracts during sporulation.

Immunoblot analysis was carried out using anti-enoylpyruvate transferase antibody. Fig. 4 showed the result of Western blot of the cell extracts during sporulation. No band was detected in the cell extracts of exponentially growing cells, but one band began to appear from t_5 and was weakly developed at t_{10}, which was roughly consistence with the variation in the activity of the transferase during sporulation.



1. Marker
2. Enoylpyruvate transferase

Fig. 3. SDS-PAGE of purified enoylpyruvate transferase on 12.5% gel.

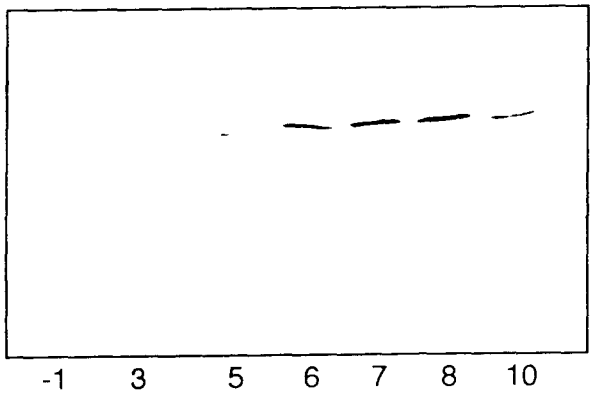


Fig. 4. Immunoblot analysis for the mother cell cytoplasmic fraction. Lane number indicates Tn. Anti-transferase antibody was prepared from sporulation phase, which was secondly reacted with peroxidase labeled goat anti rabbit IgG.

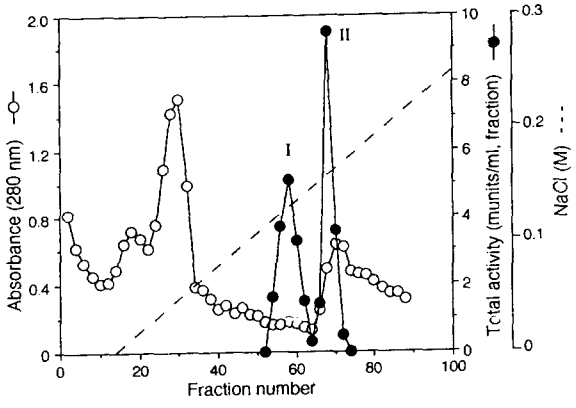


Fig. 5. Elution profile of the crude enzyme mixture at T_1 and T_7 by DEAE-Sepharose CL-6B column(11.0 × 10 cm). Same amounts of protein were applied to the column which had been equilibrated with 20 mM Tris-HCl, pH 0.8, containing 4 mM dithiothreitol.

Separation of enoylpyruvate transferase isozymes.

It was found that the transferase in exponential growth has different antigenic specificity from that in sporulation by immunoblot analysis. When the mixture of the crude enzyme in the cells at t_1 and t_7 was separated on a DEAE-Sepharose column, two transferase activity peaks, (I) and (II) were observed (Fig.5), but the fractions of both peaks were positively reacted with the anti-transferase antibody. Fig. 6 showed the individual elution profile of the crude enzyme at t_1 and t_7 by chroma-

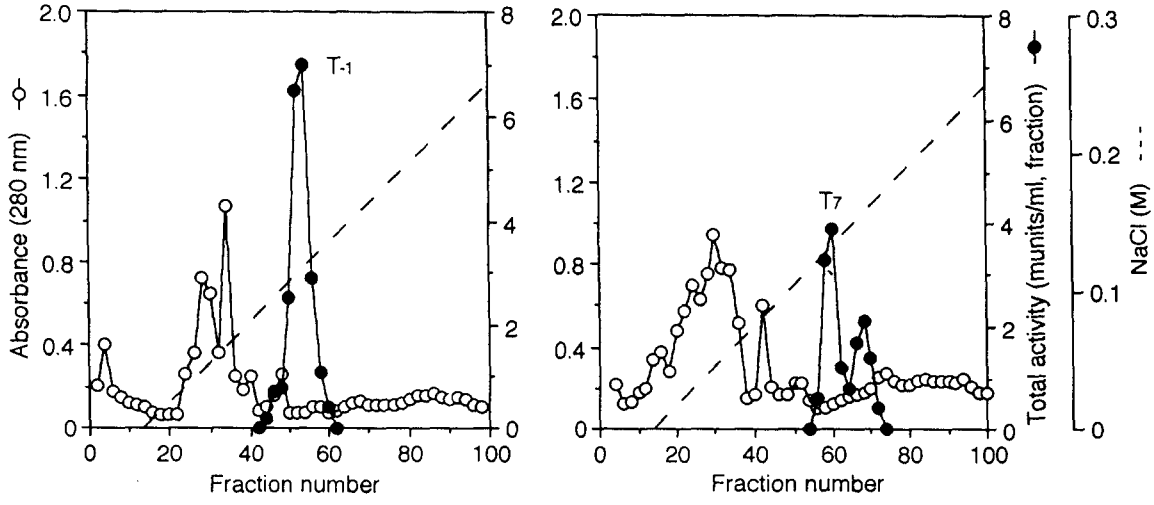


Fig. 6. Individual elution profile of the crude enzyme at T_{-1} and T_7 by DEAE-Sepharose CL-6B column. Solid circles represent total activity of enoylpyruvate transferase. Sodium chloride was used for elution, pH 8.0 with a linear gradient from 0 to 0.25 M, and the protein was monitored at 280 nm(○).

tography on the same column, which indicated that the peak (I) contained two transferase in the cells both at t_{-1} and t_7.

Discussion

Deutsher and Kornberg[2] classified eight enzymes of *B. subtilis* to three groups by examining the variation of the activity during sporulation, but no enzymes was found to reveal such a pattern as enoylpyruvate transferase, *i.e.*, decrease after the initiaton of sporulation followed by an increase at the later sporulation stage. Tipper and coworkers[4,5,16] reported that the variation in the specific activity like transferase was observed in the enzymes, : L-alanine, : D-glutamate, and D-Ala-D-Ala ligases, involved in synthesizing the UDP-GlcNAc-penta peptide precursors, but the total activity of these enzymes did not decrease during sporulation in *B. sphaericus*. Moreover, they indicated that these enzymes were controlled at the level of transcription by treating sporulation cells with antibiotic inhibitors of RNA and protein synthesis. But, little was known about each enzyme protein. Antibody specificity and the elution profile on DEAE Sepharose revealed that *B. megaterium* has at least two transferase isozymes, and each isozyme appeared specifically in the growing and in sporulating cells, respectively.

Transferase II was less stable than transferase I for storage at -20℃ and the sensitivity to 0.06 mM fosfomycin was slightly different, *i.e.*, 70% inhibition for transferase I and 29% for transferase II, respectively (data not shown). In two instances, enoylpyruvate transferase have been purified and characterized, *e.g.*, in *Staphylococcus epidermidis*[17] and in *Enterobacter cloacae*,[18] comparing the properties of the transferase from *B. megaterium* at sporulation phase with those from the above sources. There are only a few reports of the isozymes found

in cell extracts not in spores during sporulation in *Bacillus* species. Agrawal and coworkers[1] reported early and later aconitases in *B. cereus* and Paulus and coworkers,[12] carbamyl phosphate synthetase P and A in *B. subtilis*, respectively, but definite physiological functions of these isozymes was not elucidated. While, Heilmann and coworkers indicated the existance of at least two independent glucose dehydrogenase genes in *B. megeterium* M 1286.[8] Isozymes are also synthesized for common reactions in branched anabolic pathways.[1] Paulus and coworkers,[14, 19] and Graves and Switzer[6] reported that *B. subtilis* has three aspartokinases and the specific activity of aspartokinase I was nearly constant under all phases of growth, while apartokinase II declined rapidly during stationary phase. They mentioned that aspartokinase I is to provide precursors for the synthesis of diaminopimelate and dipicolinate during sporulation and aspartokinase II has a role in providing precursors for lysine, methionine and threonine during periods of rapid protein synthesis. As it was indicated that cell wall turnover is negligible during the stationary phase and during most of the sporulation phase in *B. megaterium*,[3] it seems to be reasonable that the activity of each transferase isozyme increase only when UDP-GlcNAc is metabolized to synthesized two types of peptidoglycans.

Immunoblot analysis with anti-transferase antibody indicated that an increase in activity was parallel to the induction of enzyme protein synthesis during sporulation in similar manner as UDP-GlcNAc 4-epimerase, suggesting that sporulation specific transferase is regulated at the level of transcription or translation. In *B. sphaericus*, the chemical composition of vegetative cell wall peptidoglycan is quite different from that of cortex peptidoglycan *i.e.*, the former is devoid of DPM and the latter of lysine, respectively, and it was demonstrated that two enzymes, UDP-GlcNAc-L-Ala-D-Glu : Lys ligase and the equivalent meso-Dpm ligase switch the pathway of synthesizing each peptidoglycan precursors.[4] It is still unknown that enzyme switches the pathway of metabolizing UDP-GlcNAc to synthesize each peptidoglycan in *B. megaterium*, but a different mechanism such as a regulatory control by a membrane-associated enzyme might be possible. Many important questions, *e.g.*, the regulation of each transferase isozyme and the switching mechanism of the pathway remain unanswered and further work is need.

References

1. Agrawal, P. K., Garg, G. K., and Gollakota, K. G., *Biochem. Biophys. Res. Commun.*, **70**, 987-960(1976).
2. Deutscher, M. P., and Kornberg, A., *J. Biol., Chem.*, **243**, 4653-4660(1968).
3. Doyle, J., Chaloupka, J., and Vinter, V., *Microbiol. Rev.*, **52**, 556-567(1988).
4. Linnett, P. E., and Tipper, D. J., *J. Bacteriol.*, **120**, 342-354(1974).
5. Linnett, P. E., and Tipper, D. J., *J. Bacteriol.*, **125**, 565-574(1976).
6. Graves, L. M., and Switzer, R. L., *J. Bacteriol.*, **172**, 218-223(1990).
7. Gunetileke, K. G., and Anwar, R. A., *J. Biol. Chem.*, **243**, 5770-5778(1968).
8. Heilmann, H. J., Magert, H. J, and *Gassen, H. G., Eur. J. Biochem.*, **174**, 485-490(1988).
9. Imagawa, M., Oku, Y., El-Belbasi, H. I., Teraoka, M., Nishinara, T., and Kondo, M., *Microbiol. Immunol.*, **29**, 1151-1162(1985).
10. Koshikawa, T., Beaman, T. C., Pankratz, H. S., Corner, R. C., and Gerhart, P., *J. Bacteriol.*, **159**, 624-632(1984).
11. Nishikawa, J., Iwawaki, H., Takubo, Y., Nishihara, T., and Kondo, M., *Microbiol. Immunol.*,

30, 1085-1093(1986).

12. Paulus, T. J., and Switzer, R. L., *J. Bacteriol.*, **137**, 82-91(1979).
13. Pitel, D. W., and Gilvarg, C., *J. Biol. Chem.*, **245**, 6711-6717(1970).
14. Rosner, A., and Paulus, H., *J. Biol. Chem.*, **246**, 2965-2971(1971).
15. Takubo, Y., Atarashi, M. Nishihara, T., and Kondo, M., *Microbiol. Immunol.*, **32**, 973-979 (1988).
16. Tipper, D. J., and Pratt, I., *J. Bacteriol.*, **103**, 305-317(1970).
17. Wickus, G. G., and Strominger, J. L., *J. Bacteriol.*, **113**, 287-290(1973).
18. Zemell, R. I., and Anwar, R. A., *J. Biol. Chem.*, **250**, 3185-3192(1975).
19. Zhang, J., Hu, F., Chen, N., and Paulus, H., *J. Bacteriol.*, **172**, 701-708(1990).

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생명과학의 편집계획과 관련된 원고들은 수시로 모집하오니, 회원동정에 관한 부분까지도 본 연구회 사무실로 보내주시기 바랍니다.

편집은 권두언, 총설, 최근연구동향, 세미나리포트, 학회참관기, 생명과학중계실, 생명과학에세이, 오륙도 게시판, 자유 칼럼 순으로 계획되어 있으니 관련원고를 적극적으로 투고하여 주시면 보다 충실한 생명과학지로 성장할 수 있을 것으로 믿습니다.

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