Glycine Effect on Spheroplasting and Nodule Bacteroids of *Rhizobium japonicum*

Sung-Hoon Kim, Chang-Jin Kim, Yoon Rhee, Ick-Dong Yoo, and Tae-Ick Mheen

Microbial Technology Lab., Genetic Engineering Center, KAIST, Seoul, Korea

*Rhizobium japonicum*원형질체 형성과 근류 Bacteroids에 미치는 Glycine의 영향

김성훈 · 김창진 · 이 윤 · 유익동 · 민태익

한국과학기술원 유전공학 센터

Different spheroplasting methods using glycine were tried to fast and slow-growing *R. japonicum*. Although one of the fast growers, R-271 showed normal growth in the presence of 4mg/ml glycine, cell morphology and colony forming unit (CFU) were greatly different from the cells of late log phase grown in the medium without glycine. In parallel, R-271 became sensitive to lysozyme after 6hr incubation in medium containing glycine (3.5mg/ml). After 24hr cultivation in glycine (100 μg/ml) medium, one of the slow growers, R-214 was also susceptible to lysozyme action. Spheroplasting frequency of both strains was over 96% by glycine and lysozyme. Spheroid cell was also found in bacteroids from root nodule and soluble glycine content was relativiely smaller than other amino acids in soybean nodule extracts.

Rhizobium transforms into bacteroid in root nodule, which differs from its free living state in physiology (Bergersen, 1974) and morphology. Among the bacteroidal pleomorphism appeared in root nodule (Carlyle, 1984), spheroid type cell is observed commonly (Berry, and Atherley 1984[a]) and it was suggested that *Rhizobium* cell wall/membrane undergoes significant changes during establishment of the root nodule symbiosis (Bal and Shantharam, 1980). Meanwhile, it was reported that *Rhizobium* became transformable by glycine (Drozanska, *et. al.*, 1982), and some *Rhizobium* assumed "Bacteroid-like" form in the glycine enriched media (Van Eqeraat, 1976). Besides, considering that glycine is widely used as murein synthesis inhibitor in Gram negative bacteria (Martin, 1983), it could be expected that glycine be a common factor in spheroplasting and bacteroid morphogenesis of *Rhizobium* species.

This suggestion was recently supported by the report which described structural similarity between glycine induced spheroplast and bacteroid in root nodule of slow-growing *R. japonicum* (Berry and Atherly, 1984[a]). In this research, spheroplasting of fast-and slow-growing *R. japonicum* isolated in this laboratory was tried using glycine and morphological change was compared with bacteroid.

MATERIALS & METHODS

Media and Strains

Fast-and slow-growing *R. japonicum* used in this research were isolated from domestic soybean

field and *R. japonicum* USDA 191 and USDA 110 rif^r were kindly provided by Dr. R.C. Valentine (U.C. Davis, U.S.A). Yeast extract-mannitol (Broughton, 1976), Bactopeptone (Hirsh *et al.*, 1980) and Bactotrypton-yeast extract (Berry and Atherly , 1984[a]) media were selectively used for cultivation of *R.japonicum.*

Glycine resistance of *R. japonicum*

Each strain was inoculated at 2% level into yeast extract-mannitol (YM) broth containing various concentration of glycine and the growth was determined spectrophotometrically at 660nm after 12hr cultivation for fast growers and 48hr for slow growers.

Spheroplasting of fast-growing *R. japonicum*

The cells of fast-growing *R. japonicum* R-271 which was sensitized to lysozyme action by cultivation in glycine enriched Bactopeptone (PA) broth, was harvested by centrifugation at 5000 rpm for 10min. They were resuspended in 2ml of PA broth containing lysozyme 100 ug/ml, 10mM EDTA, 14% sucrose (pH 7.4) and incubated for overnight at 28°C for shperoplasting.

Spheroplasting of slow-growing *R. japonicum*

Berry and Atherly's spheroplasting method (1984[a])was applied basically for slow growing *R. japonicum* R-214 except glycine and lysozyme concentration, which was adjusted for each strain.

Electron Microscopy

Spheroplast was observed through electron microscope (Hitachi HU125C) directly after dipping grid into spheroplasting suspension at below 10000 magnitude and at higher magnitude, spheroplast was fixed with glutaraldehyde and osmium (Cole and Porkin, 1981), then observed.

Amino acids analysis of nodule extract

Collected nodules (approximately 25g) were washed with 70% ethanol and double distilled water several times. After squashed in mortar. 150ml double distilled water was added and mixture was shaken overnight at room temperature. Insoluble solid of the extract was cleared through filtration and centrifugation. Final cleared solution was concentrated into about 1/10 volume by vacuum rotary evaporation. Concentrate was finally filtered through milipore filter and soluble amino acids composition was analysed using amino acid analyser (Beckman Model 116). The amount of protein in nodule extract was determined by Lowry method.

RESULTS & DISCUSSION

Glycine resistance of *R. japonicum*

Glycine resistance of several fast-and slow-growing *R. japonicum* was determined since glycine may have inhibitory effect to rhizobial growth (Carlyle, 1984). As shown in table 1, each *Rhizobium* strain had different glycine resistance. Among fast-growers, R-271 showed specifically high resistance. Fast grower, R-271 and another high nitrogen fixing strain R-214 among the slow-growers were chosen as sample strain in this research.

Glycine effect on cell growth and morphology

R-271 showed normal growth in PA broth containing glycine 4mg/ml except a little extended lag phase (Fig. 1). But CFU in the glycine enriched media was decreased to below 10% of control since cell growth passed late exponential phase (Fig. 2) meaning that cell might become osmotically sensitive owing to disturbed murein synthesis by the presence of glycine and/or cell morphology might be changed to result in decreasing

Table 1. *Glycine resistance of R.japonicum*

Strain	Fast-growing group						Slow-growing group					
	R-41	R-73	R-247	R-271	R-289	USDA 191	R-67	R-138	R-214	R-224	R-256	USDA 110rif^r
Glycine* (mg/ml)	0.1	0.5	0.2	4	0.1	0.1	0.05	0.05	0.1	0.1	0.1	0.3

* Glycine concentration described in table was the highest dose at which each strain showed normal growth compared to control without glycine.

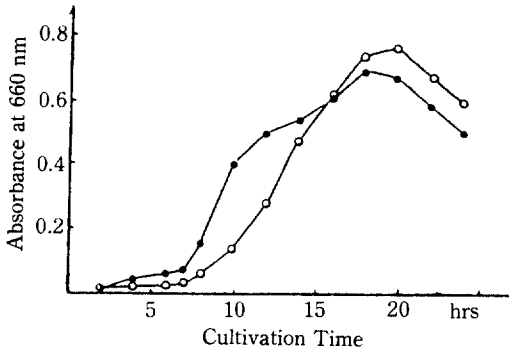

Fig. 1. *Growth curves of R-271 in the prescence of glycine*
-○-○- PA containing glycine 4mg/ml medium
-●-●- PA medium

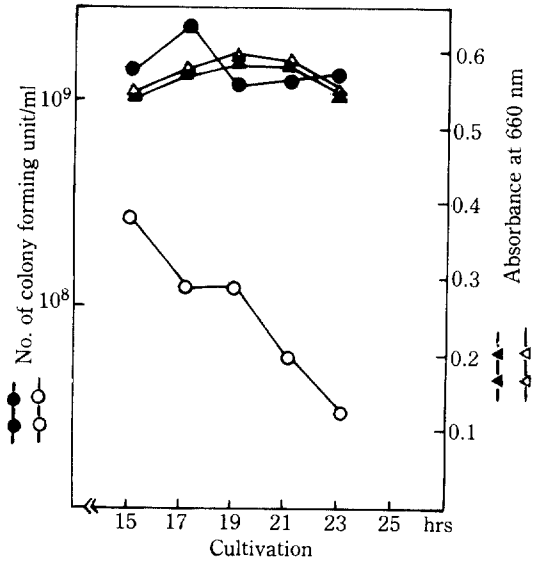

Fig. 2. *Cell growth and colony forming unit in prescence and absence of glycine*
(○, △) PA containing glycine 4mg/ml medium
(●, ▲) PA medium

CFU. Cell morphology was gradually changed with cultivation time in glycine enriched media and finally spheroid type cell began to appear in culture broth (Fig. 3) but most of them seemed to be ghosts or dead cells (Fig. 4). Therefore, glycine treating time needed to be decreased to improve shperoplast viability. Other amino acids did not show any effect on cell morphology at the highest concentration which did not inhibit the cell growth (unpublished data).

Spheroplasting of fast-growing *R. japonicum*

Although cell growth was normal in the medium containing 4mg/ml of glycine (Fig.1), cell became too fragile to be manipulated in viable state for spheroplasting. Glycine concentration was readjusted, accordingly,for spheroplasting into 3.5mg/ml, at which cell showed also normal growth and was more tolerant mechanically and osmotically. R-271 became sensitive to the action of lysozyme after 6hr growth in PA broth containing 3.5 mg/ml glycine (Fig.5). Spheroplasts were observed after over-night incubation at 28°C and the frequency was over 96%. In parallel, spheroplasting of other fast-growers including USDA 191 were also tried following similar method described above and it was proved as generally efficient spheroplasting method for fast-growing *R. japonicum*.

Spheroplasting of slow-growing *R.japonicum*.

Since spheroplasts of slow-growing *R. japonicum* were already successfully prepared by glycine and lysozyme treatment (Berry and Artherly, 1984[a]), this method was applied to the slow-

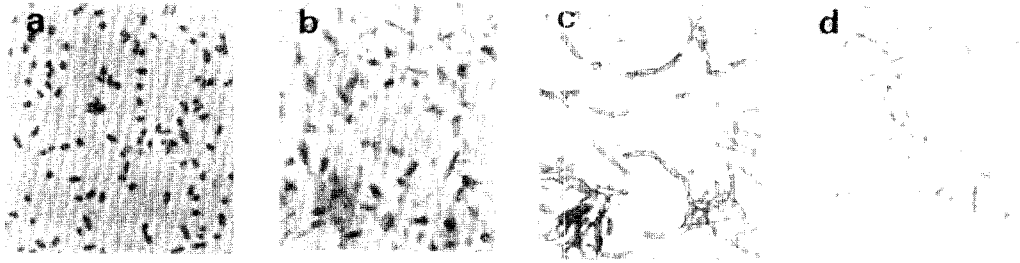

Fig. 3. *Changes of cell morphology of R. japonicum R-271 with cultivation time in PA broth containing glycine*
a. Normal cell morphology (0 hr).
b. Enlarged cell (4 hr).
c. Cell length was enlarged almost to maximum (6-12hr).
d. Spheroid cell (18 hr).

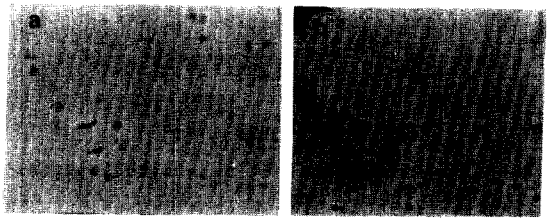

Fig. 4. *Spheroplast of R. japonicum R-271 induced by glycine and lysozyme.*
a. Ghost cells of R-271 in the glycine enriched PA media.
b. Spheroplast of R-271 from lysozyme and glycine treatment.

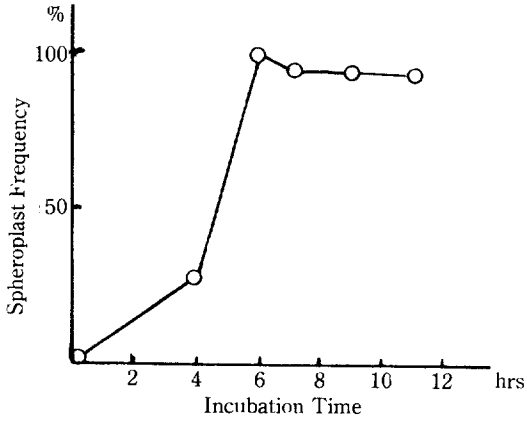

Fig. 5. *Glycine effect on spheroplasting frequency R. japonicum R-271 culture broth was PA containing glycine (4 mg/ml)*

growing strains with slight modificaton resulting in spheroplasting of over 95% (Fig.6).

Electron microscope of *R. japonicum* spheroplast

Spheroplasts of fast- and slow-growing *R. japonicum* were observed through electron

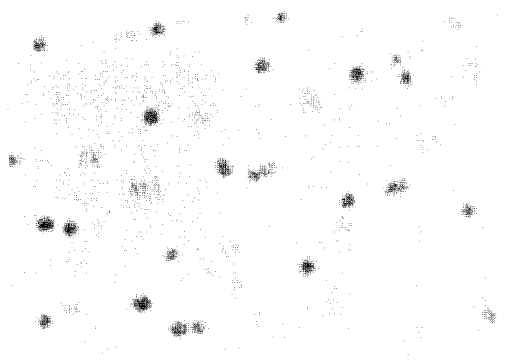

Fig. 6. *Spheroplast of R. japonicum R-214 (x2000, Phase contrast microscope)*

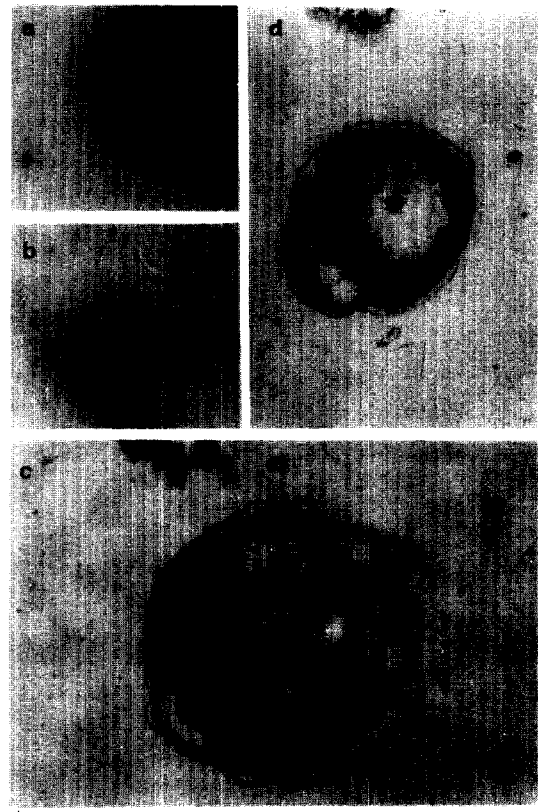

Fig. 7. *Electron microscope of spheroplast of R. japonicum R-271 and R-214*
a. *R. japonicum* R-271 (X6400)
b. *R. japonicum* R-271 (X7700).
c. *R. japonicum* R-271 (X40000)
d. *R. japonicum* R-214 (X40000)

microscope. Fig. 7 a and b showed intact spherical cells in lysozyme broth. Spheroplast is very useful for transformation (Hopwood, 1981), cell fusion (Peberdy, 1980) and isolation of membrane protein etc. Transformation using spheroplast of slow-growing *R. japonicum* (Berry and Artherly, 1984[b]) and *R meliloti* (Jung *et. al.*, 1984) was already reported. From Fig. 7 c and d, it was recognized that spheroid cell of R-271 and R-214 still retained large amount of their outer membrane which makes it necessary that spheroplasting condition described here be improved further to remove larger part of outer membrane since spheroplast made here could be possibly used for transformation of foreign DNA but insufficient for cell fusion. Now improvement is being tried in parallel with the establishment of

cell regeneration conditions.

Morphology of bacteroid from root nodule

In nodule, *Rhizobium* is known to take swollen and either globular,ellipsoidal, club shape or branched type among which swollen spherical cell types, as seen in Fig. 8 b and c, are commonly found in almost all soybean root nodule isolates in this laboratory. They are also found in the glycine enriched culture broth as in Fig. 8, suggesting glycine role in this phenomenon and structural similarity between them, and this fact is also supported by Van Eqeraat (1976). Spherical bacteroids are also clearly demonstrated in Berry and Atherly's report (1984[a]). In other respect,differential refractivity between swollen spheroid and rod type cells observed in phase contrast microscope was noteworthy, suggesting structural difference of cell walls which is consistent with the results of Bergersen and Brigg's (1958) and Ching *et. al's* (1977). Each type of cells in nodule isolate were separately shown in Fig. 8 d,e and f.

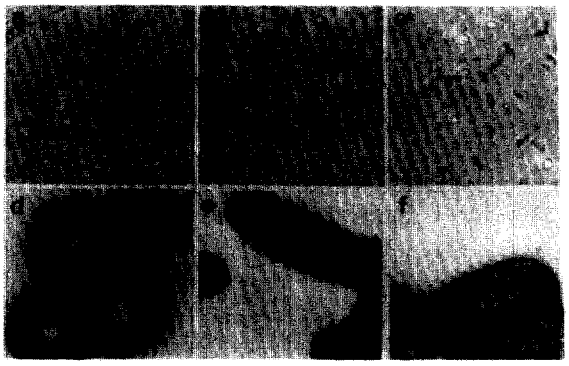

Fig. 8. *Spherical cells detected in glycine enriched culture broth and nodule isolates*
a: USDA191 in glycine enriched media (x800)
b: USDA191 in nodule isolate (x800)
c: R-214 in nodule isolate (x800)
d: Spherical cells in nodule isolate of USDA191
e: Intermediate type cells in nodule isolate of USDA191
f: Rod type cells in nodule isolate of USDA191
d.e.f. were observed through electron microscope (x15000)

Amino acids analysis of soybean root and nodule extract

Free amino acid of soybean root and nodule extract was analysed. As shown in table 2 there was not qualitative difference in amino acid composition between two but they showed different quantitative pattern among free amino acids. Especially, it is noteworthy that glycine was present second to minimum after aspartic acid in nodule extract although it was existed in relatively larger amount in root extract. This fact is suggesting, even if not directly, the possibility that glycine might be involved in or conjugated into bacteroidal cell wall in root nodule, from which it could not be detected as free amino acid.

Table 2. *Amino acid composition of soybean root and nodule extract.*

Amino acids (mg/100m*l*)	Sample	
	Nodule extract	Root extract
Lysine	2. 18	0. 22
Histidine	0. 59	0. 05
Arginine	1. 15	–
Aspartic acid	Trace	–
Threonine	0. 83	–
Serine	1. 76	–
Glutamic acid	0. 51	0. 23
Proline	0. 73	–
Glycine	0. 42	0. 48
Alanine	4. 04	0. 89
Valine	1. 98	–
Methionine	0. 45	0. 04
Isoleucine	0. 88	0. 14
Leucine	2. 70	0. 60
Tyrosine	0. 87	0. 23
Phenylalanine	1. 57	0. 44
Tryptophan	0. 43	–
Total protein	230	–

"–" means "Not determined".

적 요

Glycine이 *R. japonicum*의 spheroplasting에 미치는 영향을 검토하였다. Fast-growing *R. japonicum* R-271은 4 mg/m*l*의 glycine 함유배지에서 생육이 정상이었으나 세포 형태는 배양시간에 따라 변화하였으며 colony 형성

단위는 log-phase 이후 현저히 감소하였다. R-271은 3.5mg/ml glycine 함유배지에서 6시간 배양후에, slow grower R-214는 0.1mg/ml glycine 함유배지에서 24시간 배양후에 lysozyme의 작용을 받았다. 두 균주 모두 이러한 처리에 의해 96% 이상의 spheroplast 형성빈도를 나타내었다. 한편 spheroid-type cell은 대두근의 nodule에서 분리한 bacteroid에서도 관찰되었으며 nodule extract에 존재하는 유리 amino acid 중 glycine은 다른 amino acid에 비해 소량 존재하고 있었다.

ACKNOWLEDGEMENT

I am very grateful to Dr. Soon Hyung Lee in the Institute of Agricultural Science (Suweon, Korea) for his kind help in electon microscopy and Miss In-Ja Ryu for her technical assistance.

REFERENCES

1. Bal, B.K. and S. Shantharam, 1980: Changes in the outer wall of *Rhizobium* during development of root nodule symbiosis soybean. *Can. J. Micrbiol.*, **26,** 1096-1103
2. Bergersen, F. J. and M.J. Briggs, 1958.: Studies in the bacterial component of soybean root nodules: cytology and organization of host tissues. *J. Gen. Microbiol.*, **19,** 482-490
3. Bergersen, F.J., 1974.: In *"The Biology of Nitrogen Fixation"* ed. by A. Quispel. North-Holland Pub. Co. Amsterdam 47-495.
4. Berry, J.O. and A.G. Atherly, 1984[a].: Spheroplasts of *R. japonicum*. *Can. J. Microbiol.*, **30, 415-419**
5. Berry, J.O. and A.G. Atherly, 1984[b].: Induced plasmid genome rearrangements in *R. japonicum*. *J. Bacteriol.*, **157,** 218-224.
6. Broughton, W.J., 1976.: In *"Symposium Soil Microbiology & Plant Nutrition"* 1-24.
7. Carlyle, J.D., 1984.: In *"Bergey's manual of systematic bacteriology"* ed. by N.R. Krieg Williams & Wilkins Co. 223-234
8. Ching, T.M., S. Hedtke and W. Newcomb, 1977.: Isolation of bacteria, transforming bacteria, and bacteroids from soybean nodules. *Plant Physiol.*, **60,** 771-774.
9. Cole, R.M. and T.J. Porkin, 1981.: In *"Manual of methods for general bacteriology"* ed. by. P. Gerhardt, *et. al.*, American Society for Microbiology, 40-41
10. Drozanska, D., W. Drozanski and Z. Lorkiewicz, 1982.: Effect of glycine on the transformation of *R. trifolii. Microbios,* **35**(139), 31-38
11. Hirsh, P.R., M. Van Montagu, A.W.B. Johnston, N.J. Brewin. and J. Schell, 1980.: Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *R. leguminosarum. J. Gen. Microbiol.*, **120,** 403-412
12. Hopwood, D.A. 1981.: Genetic studies with bacterial protoplasts. *Ann. Rev. Microbiol.*, **35,** 237-272
13. Jung, S.O., T.L.Huh and S.Y. Lee, 1984.: In "Studies on the Microbial Strain Development Using Gene Manipulation" KAIST Report to the MOST N50-2075-5 ed. by T.I. Mheen *et. al.* 153-188
14. Martin, M.M., 1983.: In *"Protoplasts* 1983" ed. by I. Potrykins, *et. al.* Birkhäuser Verlag, Basel, 213-226
15. Peberdy, J.F. 1980.: Protoplast fusion-a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme Microb. Technol.*, **2.** 23-29
16. Van Eqeraat, A.W.S.M., 1976.: Response of Rhizobium species and pea seedlings to glycine. *Plant Soil,* **45** (1) 191-199

(Received August 9, 1985)