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Effect of the Extraction Method on the Soybean Embryo Factor 3 Activity

Kyung-Hoon Lee[2], Dong-Hyo Chung[2], Seong-San Kim[2]
Youn-Ho Song[2] and Woo-Yeon Kim[1]*

[1]Department of Biotechnology and [2]Department of Food Science and Technology,
College of Industrial Studies, Chung-Ang University, Ansung 456-756, Korea

Abstract : Soybean nuclear extracts were prepared to detect SEF3(soybean embryo factor 3), which is presumed to be a *trans*-acting factor for the expression of the soybean β-conglycinin α' subunit gene. To increase the specific activity of DNA probe during labeling with [α-^{32}P]dATP, dATP was added to a final concentration of 1.1 mM during the chase reaction. It results in approximately four-fold increase of specific activity of the DNA probe. Effects of several modifications in preparation of soybean nuclear extracts were examined. It was found that glycerol is effective to stabilize SEF3 during the preparation of nuclear extracts and polyethylenimine could be used to increase the specific activity of SEF3 in nuclear extracts(Received January 20, 1995; accepted February 22, 1995).

Introduction

β-Conglycinin, the 7S globulin storage protein in soybean seeds, is comprised of three subunits, α, α', and β.[1] These subunits are encoded by separate genes that are expressed exclusively in maturing seeds. Expression of the genes encoding these subunits is regulated primarily at the level of transcription.[2] Although there are more copies of the genes encoding the subunit than those encoding the α' or α subunit,[2-4] these polypeptides accumulate to similar levels in mature embryos.[5,6] This suggests that individual α or α' subunit genes are expressed at higher levels than are genes which encode the β subunit. This hypothesis is supported by results from the experiment in which individual genomic clones of the α' and β subunit genes were expressed in transgenic plants.[6] There could be some specific upstream sequences that are present only in α' subunit gene.

Tissue specific and temporally regulatory enhancer sequences of the α' subunit gene have been localized between −257 and −69 bp relative to the transcription initiation site by deletion analyses of the 5'-flanking sequence of the α' subunit gene in the transgenic plant.[7] Transcriptional regulation in eukaryotes generally is related to the binding of *trans*-acting factors to regulatory sequences within a gene that affects the frequency of transcription initiation.[8,9] For this reason, researches on transcriptionally regulated genes have been focused on identifying such *trans*-acting factors. Two factors were identified that interact with specific sequence elements within this region. One factor, SEF3(soybean embryo factor 3), which only interacts with upstream region of α' subunit gene but not with that of β subunit gene, binds exclusively to a region composed of two elements located at −183 to −169 bp and −153 to −134 bp relative to the transcription initiation site.[10] Each of these sites includes the hexanucleotide sequence AACCCA, which may serve as a primary recognition sequence.

In the previous reports[11,12] SEF3 was identified using the DNA fragment SE3 (5'-GATCTCACCAACTC**AACCCA**TCATGAGCCCACACATTTGTTGTTTCT**AACCCA**ACCTCAAACG-3') as a probe and characterized. In this report several extraction methods were examined to increase the activity of SEF3 extracted from soybean seeds.

Materials and Methods

Materials

E.coli DNA polymerase I, Klenow fragment was purchased from KOSCO Biotech. *Eco*RI, *Hin*dIII and *Bam*HI restriction enzymes were from KOSCO Biotech. Leupeptin hemisulfate, poly[d(I-C)]:poly[d(I-C)] and other chemicals were obtained from Sigma. [α-^{32}P]dATP was purchased from Amersham. *E. coli* DH5αF' was used for the plasmid production.

Nuclear extracts

Nuclei were prepared from immature soybean seeds (*Glycine max* cv Provar) essentially as described by Allen

Key words : β-conglycinin α' subunit, soybean embryo factor
*Corresponding author

et al.[10] All extraction steps were performed at 4℃. Embryos(50 g) 30 days after pollination were frozen in liquid nitrogen and grounded to a fine powder with Waring Blendor. This powder was transferred to a beaker and suspended in 150 ml of buffer A(2.5% Ficoll, 5% dextran, 25 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 0.5% Triton X-100, 0.44 M sucrose, 10 mM β-mercaptoethanol, 0.2 mM EDTA, 2 mM spermine-HCl, 10 μg/ml leupeptin hemisulfate). The slurry was homogenized by three 30-sec bursts at 12,000 rpm with a Polytron PT 3000 homogenizer(Kinematica AG) and filtered through two layers of 70-μm mesh Nytex. Crude nuclei were pelleted by centrifugation at 7,000 rpm for 15 min in a Beckman JA-14 rotor and resuspended in buffer A in the absence of spermine-HCl. Nuclei were pelleted again, resuspended in 30 ml buffer C (20 mM Tris-HCl, pH 7.9, 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 10 μg/ml leupeptin hemisulfate), and subjected to Dounce homogenization(10 strokes with B pestle). The solution was centrifuged at 15,000 rpm for 30 min in Beckman JA-20 rotor, and the supernatant was dialyzed for 5 hr against buffer D (20 mM Tris-HCl, pH 7.9, 20% glycerol, 100 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10 μg/ml leupeptin hemisulfate). The dialysate was recentrifuged to remove precipitated globulin storage proteins and to float much of the remaining lipid, and the aqueous supernatant, which was defined as nuclear extract, was stored at −70℃. Soybean embryo S-100 was prepared as described by Dahmus and Kedinger.[13]

Polyethylenimine(PEI) nuclear extract was prepared as follows. Crude nuclei prepared as described above were resuspended in buffer B (20 mM Tris-HCl, pH 7.9, 25% glycerol, 0.1 M KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 10 μg/ml leupeptin hemisulfate). Nuclear proteins were precipitated by the dropwise addition of 4 μl of 10% PEI per ml of nuclear homogenate. The pellet was resuspended in buffer C and PEI nuclear extract was prepared as described in the nuclear extract preparation. Polyvinylpolypyrrolidone (PVPP) nuclear extract was prepared as described in the nuclear extract preparation except that buffer A contained 1.5% PVPP.

Cell extract precipitated with ammonium sulfate (CEAS) was prepared as follows. Embryos(50 g) were frozen in liquid nitrogen and grounded to a fine powder with Waring Blendor. This powder was transferred to a beaker and suspended in 150 ml of buffer E (25 mM Tris-HCl, pH 7.4, 20 mM KCl, 1 mM EDTA, 5 mM EGTA, 2.5 mM $MgCl_2$, 5% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin hemisulfate). The slurry was homogenized by three 30-sec bursts with a Polytron and filtered through two layers of 70-μm mesh Nytex. Glycerol was added to a final concentration of 10%. Proteins and nucleic acid were precipitated by the addition of solid ammonium sulfate (0.2 g/ml of solution) and 1N NaOH (1 μl/ml of solution) is added. The suspension was stirred for 30 min and centrifuged at 15,000 rpm for 30 min. SEF3 was precipitated by the addition of solid ammonium sulfate(0.17 g/ml) to the supernatant. The suspension was stirred for 30 min and centrifuged at 15,000 rpm for 30 min. The pellet was dissolved in 20 ml of buffer D and dialyzed against 1000 ml of buffer D.

Ammonium sulfate precipitation

SEF3 was precipitated by the slow addition of 0.2 g solid ammonium sulfate per ml of solution from the nuclear extract, PVPP nuclear extract, and PEI nuclear extract, respectively. The slurry was stirred for 30 min and centrifuged at 15,000 rpm for 30 min and the solid attached to the tube or floated on the surface was resuspended in 5 ml of buffer D and dialyzed against 1000 ml of buffer D.

DNA probe

SE3 oligonucleotide and a complementary oligonucleotide, which makes the annealed DNA contain the *Bgl*II site at the 5' end and the *Bam*HI site at the 3' end after annealing with the above two oligonucleotides, were synthesized. The annealed DNA was subcloned into the *Bam*HI site of pUC19 and the plasmid was named pSE3. The plasmid pSE3 was digested with *Eco*RI and *Hind*III. The ends of the fragments were filled in with [α-^{32}P] dATP(3,000 Ci/mmole), dCTP, dGTP, and dTTP using Klenow fragment of DNA polymerase I. The labeling reaction mixture was loaded on 6% polyacrylamide gel and run in 1 X TBE buffer at 100 V. The small fragment, which contains two AACCCA hexanucleotides, was electroeluted and used for DNA probe.

Gel mobility shift assay

Binding reactions were performed in a volume of 20 μl containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 μg poly[d(I-C)]:poly[d(I-C)], and 5 μl of nuclear extract. This mixture was incubated for 10 min at room temperature, and then 0.5 ng to 1 ng of probe(5,000 to 10,000 cpm) was added, and incubation continued for an additional 15 min. After incubation, the reaction mixtures were loaded on 4% polyacrylamide gels and run in 0.5 X TBE buffer at 100 V. Gels were fixed, dried, and exposed to X-ray film with an intensifying screen overnight at −70℃.

Results and Discussion

Preparation of SE3 probe

The plasmid pSE3 was digested with *Eco*RI and *Hin-*

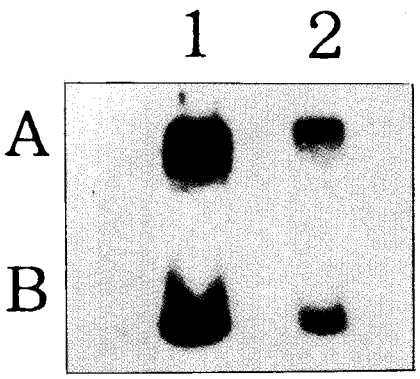

Fig. 1. Effect of addition of dATP on the incorporation of [α-^{32}P]dATP into SE3 probe after the first labeling reaction. Lane 1, addition of 1 mM dATP after the first reaction; lane 2, no addition; A, plasmid without SE3 fragment; B, SE3 fragment.

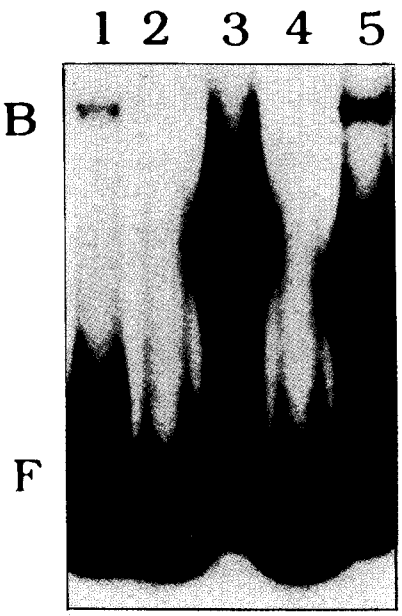

Fig. 2. Glycerol effect on SEF3 activity during the preparation of soybean nuclear extract. Lane 1, standard nuclear extract prepared in the presence of glycerol; lanes 2 and 4, S-100; lanes 3 and 5, nuclear extracts; lanes 2 and 3, samples prepared in the absence of glycerol; lanes 4 and 5, samples prepared in the presence of glycerol; B, binding complex; F, free probe.

*d*III and labeled as described in the Methods. The labeling mixture was incubated for 30 min at 23℃ in a 20-μl reaction volume containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT, 5 units of DNA polymerase I Klenow fragment, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 0.1 mM dATP, 20 μCi [α-^{32}P]dATP, and approximately 0.5 μg of pSE3 digested with *Eco*RI and *Hin*dIII. After the first reaction was finished, dATP was added to a final concentration of 1.1 mM and the reaction continued for an additional 30 min. Fig. 1 shows the effect of dATP addition after the first reaction. The addition of 1mM dATP(lane 1) during the chase reaction

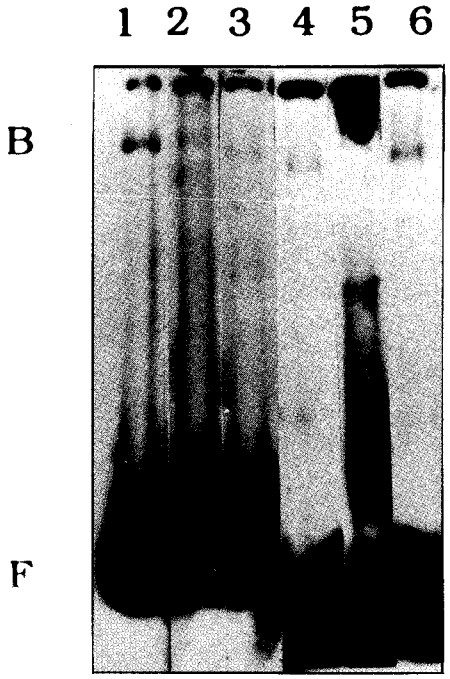

Fig. 3. SEF3 binding activities of the various nuclear extracts. Lane 1, nuclear extract; lane 2, nuclear extract precipitated with ammonium sulfate; lane 3, cell extract precipitated with ammonium sulfate(CEAS); lane 4, nuclear extract prepared with PVPP and precipitated with ammonium sulfate; lane 5, nuclear extract prepared with PEI; lane 6, nuclear extract prepared with PEI and precipitated with ammonium sulfate.

increased the incorporation of [α-^{32}P]dATP about four-fold compared to the reaction without additional dATP (lane 2). It appears that the concentration of dATP in the first reaction is much lower than the Km value of DNA polymerase I for dATP.

SEF3 binding activity with various nuclear extracts

Fig. 2 shows the glycerol effect on SEF3 activity during the preparation of nuclear extract from soybean embryos 30 days after pollination. Addition of glycerol to buffer C during the preparation of nuclear extract(lane 5) clearly increased the SEF3 activity compared to the nuclear extract prepared in the absence of glycerol(lane 3). This implies that SEF3 is stabilized in the presence of glycerol. Because soybean embryo S-100 prepared both in the absence and presence of glycerol did not show SEF3 activity in the case of soybean embryos 30 days after pollination(see lanes 2 and 4), nuclear extract was used for further experiments. To increase the SEF3 activity in nuclear extract, some modifications for the preparation of nuclear extract were tried. Lane 1 of Fig. 3 shows the SEF3 activity of standard nuclear extract. Lanes 2, 3, and 4 contain the various extracts precipitated with ammonium sulfate. There is no significant diffe-

rence among nuclear extract(lane 2), cell extract(lane 3), and nuclear extract prepared with PVPP(lane 4), which was added to remove phenolic compounds during the preparation of nuclear extract. In order to separate nuclear proteins from storage proteins, PEI, which precipitates DNA-DNA binding protein complex, was used during the preparation of nuclear extract. Because residual PEI binds to radioactive probe, major retardation complex was stuck in the well of the lane. It appears that residual PEI could be removed during the ammonium sulfate precipitation step and SEF3-SE3 complex was visualized clearly as shown in lane 6. PEI step reduced the amount of storage proteins in nuclear extract to a certain extent (data not shown). In the future, the development of procedure for the preparation of nuclear extract containing more active SEF3 and less storage proteins could accelerate the purification and characterization of soybean embryo factor 3.

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추출 방법에 따른 대두 배인자 3 역가

이경훈[2], 정동효[2], 김성산[2], 송윤호[2], 김우연[1]*([1]중앙대학교 생물공학과, [2]식품공학과)

초록 : 본 실험에서는 대두 β-conglycinin의 α' subunit 유전자의 발현에 관여하리라 추정되는 대두 배인자 3(SEF3) 역가를 가지는 대두 핵 추출물 조제 방법에 대하여 조사하였다. 역가 조사를 위한 DNA의 표지 반응 후에 추가로 1 mM dATP를 첨가하여 반응을 지속한 경우가 첨가하지 않은 경우 보다 약 4배 높은 방사능 역가의 DNA 탐침을 얻을 수 있었다. 이러한 DNA 탐침을 이용하여 핵 추출물 조제 방법에 따른 대두 배인자 3의 역가를 gel mobility shift assay로 조사한 결과, 조제 중 glycerol 첨가가 배인자 3을 안정화시켰고, polyethylenimine을 이용한 대두 핵 추출물 조제법이 배인자 3의 비역가를 증가시키는 데 이용될 수 있음을 알 수 있었다.

*연락저자