KOR. JOUR. MICROBIOL, December, 1988 p. 291-297 Vol. 26, No. 4

Overproduction and Purification of Ribose- Binding Proteins from the Wild-Type, Mutant and Revertant Strains in *Escherichia coli*

Park, Soonhee[1*], and Linda L. Randall[2]
[1]Dept. of Microbiology, [2]Biochem./Biophysics Program,
Washington State Univ., Pullman, WA99164, U.S.A.

리보스 결합단백질의 대량생산을 위한 야생형 수송결합변이, 복귀변이 유전자의 클로닝과 이들 단백질의 순수정제

박순희[1]·Linda L. Randall[2]
[1]워싱턴 주립대학교 미생물학과
[2]워싱턴 주립대학교 생화학 / 생물리학과

ABSTRACT : Three alleles of *rbsB* gene, *rbsB*, *rbsB103*, and *rbsB106* from the wild type, the mutant and the revertant strain, respectively, were cloned for overproduction of proteins under the control of lambda P_L promoter. Five different species of precursor and mature ribose-binding proteins were purified to homogeneity using DEAE-Sephadex column chromatography, osmotic shock pocedure, CM-Sephadex column chromatography, and Chromatofocusing column chromaography. pI of the precursor proteins and mature proteins were determined and found to be pH 8.0 and 7.5, respectively. The purified proteins were subjected to amino acid sequencing. The results confirmed the amino acid changes deduced from the DNA sequencing.

KEY WORDS □ *E. coli*, Protein Export, Ribose-binding proteins, Purification

Many hypotheses concerning the mechanism of protein export have been proposed (Randall and Hardy, 1987). Whether interaction of the nascent protein with a proteinaceous export apparatus as suggested in the signal hypothesis or spontaneous insertion of the newly synthesized protein product into the bilayer suggested in the membrane trigger hypothesis initiates the export process, the properties of the signal sequence are critical.

It is possible that the signal sequence functions in one of the following ways. If the function of the signal sequences were to interact with the membrane, then we might see the difference in the interaction depending on export competency of the proteins. If the function of the signal sequence were to influence the conformation of completed preprotein, we might see a difference in the conformation of proteins depending on their export competencies, even though it is not clear what kind of conformational difference would be expected. If the function of the signal sequence were to interact with one or several components of the export machinery, this interaction would be affected. We can attempt to answer at least some of these questions using purified precursor proteins *in vitro*.

A signal sequence mutation *rbsB*103 accumulating unprocessed precursor ribose-binding protein in the cytoplasm and a revertant carrying another mutation, *rbsB*106, in the signal sequence that processes accumulated precursor were characterized and reported (Iida *et al.*, 1985, Park, *et al.*, 1988).

Here, we report cloning of three alleles of *rbsB*

* Current Address; Genetic Engineering Center, KAIST, P.O. Box 131 Chongryang, Seoul, Korea
This work was supported by a grant from GM29798 to Linda L. Randall.

for overproduction of precursor and mature proteins, purification of these proteins and then amino acid sequencing of the purified proteins to confirm the mutational changes on the protein level for preliminary step for conformational analysis.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this work are all derivatives of *E. coli* (Table 1). NR 69 and strain 483 are kind gifts from E. Remaut.

Materials (Chemicals and Enzymes)

Chloramphenicol, ampicillin, lysozyme, and phenylmethyl-sulfonyl fluoride were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Enzymes for recombinant DNA techniques were purchased from New England Biolabs, Inc., Beverly, Mass. or Bethesda Research Laboratories, Inc., Gaithersburg, MD. Agarose for gel electrophoresis was purchased from Bio-Rad.

Table 1. *Bacterial Strains and Plasmids*

Strain/Plasmid	Relevant genotype, Source, Comment, Reference
Bacteria	
NR69	(λ Nam7 Nam53 cI857 ΔH1) Sm^r $lacZ^{am}$ *Δbio-uvrB ΔtrpA2*(H1: Δ croR-A-J-62)
483	K12 r^{m+} (λwt) (pPLC2833)
SP114	NR69 (pSP107), this study
SP115	NR69 (pSP108), this study
SP116	NR69 (pSP109), this study
Plasmids	
pAI12	*Hind*III fragment including *rbsC*, *rbsB* and *rbsK* cloned into pACYC184 (Iida *et al.*, 1985)
pAI27	Like pAI12 but with *rbsB*103
pSP106	Like pAI27 but with *rbsB*106, this study
pPLC2833	Ampicillin resistant, P_L promoter, Remaut *et al.* (1983)
pSP107	This was constructed from cloning *rbsB* from wild type strain under the control of Lambda P_L promoter on pPLC2833, this study
pSP108	This was constructed by cloning *rbsB*103 as done for pSP107, this study
pSP109	This was constructed by cloning *rbsB*106 as done for pSP107, this study

Media

LB medium containing antibiotics were prepared as described by Miller (1972).

Biochemical Analyses

Cold osmotic shock, spheroplast formation using Tris, EDTA and lysozyme and SDS-polyacrylamide gel electrophoresis were used as described (Park, *et al.*, 1988). Several column chromatographies were carried out basically by the methods suggested by manufacturers.

Recombinant DNA Technique

Isolation of DNA, enzyme reactions, agarose gel electrophoresis and transformations were carried out basically by the methods described in Maniatis *et al.* (1982).

Cloning Three *rbsB* Alleles into pPLC2833 for Overproduction of Proteins

Each purified plasmid pAI12, pAI27, and pSP106 was digested with DNA restriction endonuclease *AccI*, then the sticky ends generated were filled using dNTP(2 mM) and Klenow fragment of DNA polymerase I. Following DNA extraction by phenol and precipitation by ethanol, the *Eco*RI linker was added to the blunt ends using T4 DNA ligase to create an *Eco*RI restriction site. The ligation mixture was digested with *Hind*III first then *Eco*RI restriction endonucleases. These digests were run on agarose gels and an approximately 2.8 kb DNA fragment that contains the *rbsB* gene was isolated by electroelution. This DNA fragment that contains the *rbsB* gene was ligated with the vector plasmid pPLC2833 that was digested with *Hind*III then *Eco*RI. The ligation mixture was used to transform strain NR69. Transformants that were resistant to ampicillin were isolated and were screened for clones that carried plasmids which overproduce the proteins (See Expression).

Expression of Proteins for Overproduction

Several transformants were grown overnight on LB media individually at 30 °C, then cultures were diluted to give approximately 5×10^7 cells/m*l*

with fresh LB medium. These cultures were grown to 1×10^7 to 1.5×10^8 cells/m*l* at 30 °C, then the temperature of culture was shifted to 42 °C for 4 hrs. 15 μl of each culture were taken at several time points after temperature shift and boiled in the presence of SDS-sample buffer then run on the 13% SDS-polyacrylamide gel to see the total proteins produced from each clone.

Purification Procedures

Strains, SP114, SP115, and SP116 were grown overnight at 30 °C in 100 m*l* LB media containing ampicillin (100 μg/m*l*) and the expression of the ribose-binding proteins was confirmed by the analysis of the protein products on sodium dodecylsulfate-polyacrylamide gels as described above. Fresh LB medium (1.5 *l*) containing ampicillin (100 μg/m*l*) was inoculated with an overnight culture to give O.D. about 0.1 at 560 nm. These cultures were grown at 30 °C until O.D. reached about 0.2 to 0.3 then the temperature of incubation was shifted to 42 °C. The cultures were harvested after growth for 4 hrs by centrifugation in a Sorvall GSA at 10 K for 15 min. The pellet was processed for osmotic shock and Tris-EDTA lysozyme lysis as described and the supernatant obtained was processed by ammonium sulfate fractionation. The fractions of interest were pooled and dialyzed overnight in several changes of the proper buffer for the next column chromatography. The preparation and running of all the columns used in this study, DEAE-Sephadex column, CM-Sephadex column and Chromatofocusing column chromatography were done by following the instructions provided by manufacturers. Protein contents were measured by Lowry assay or by UV absorption.

Protein Sequencing

Protein sequencing was done with purified proteins. Sequence analyses of the amino-terminal portion of purified proteins were performed as described by Park *et al.*, (1988).

RESULTS

Overproduction of Ribose-binding Proteins Under the Control of Lambda P_L Promoter

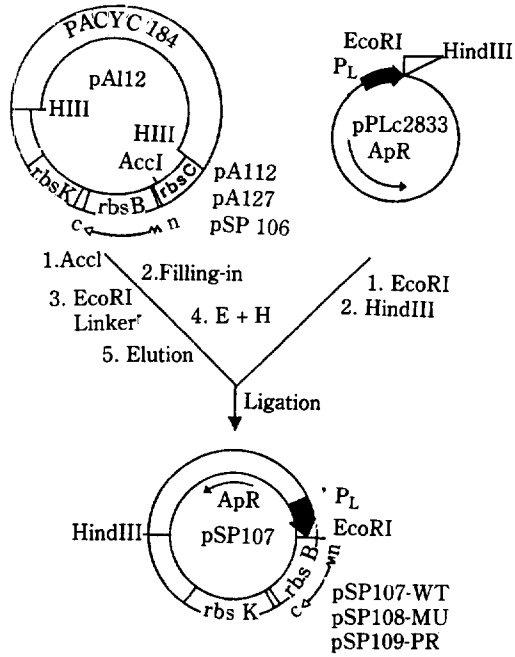

Fig. 1. *The cloning of rbsB alleles under Lambda P_L promoter control.*
Plasmids, pAI12, pAI27 and pSP106 that carried *rbsB* wild-type, mutant, and revertant genes, respectively, were cloned under the control of the lambda P_L promotor in vector plasmid pPLC2833. as described in Materials and Methods. n and c indicate the amino terminus and carboxy terminus of precursor ribose-binding protein. E and H indicate *Eco*RI and *Hind*III restriction enzyme recognition sites. ApR means ampicillin resistance.

Using the pPLC2833 vector, three *rbsB* genes were fused to the lambda P_L promoter (Fig. 1) and then transformed into the NR69 strain that has a temperature-sensitive lambda repressor. Transformants were tested for the expression of protein (data not shown).

For each of three *rbsB* genes, one clone that produced the 29 kd and/or 32 kd protein only at 42 °C compared to the control grown at 30 °C was chosen. Plasmids that contained the wild-type gene, the mutant gene or the revertant gene were referred to as pSP107, pSP108, and pSP109, respectively. The strains that carried the plasmids pSP107, pSP108, and pSP109 were referred to as SP114, SP115, and SP116, respectively. The products produced by these three strains when grown

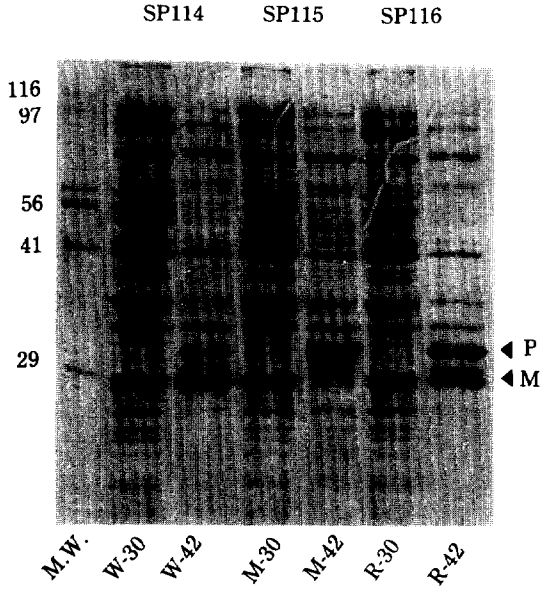

Fig. 2. *Overproduction of ribose-binding proteins from wild-type, mutant and revertant genes.*
Strains, SP114, SP115, and SP116 which carried wild-type, mutant, and revertant genes, respectively, under lambda P_L promoter control were grown and products were analyzed on SDS-polyacrylamide gels as described in Materials and Methods. The products synthesized at 30 °C (before induction) and 42 °C (after induction) were compared. W, M, and R indicate wild-type, mutant, and revertant strain. 30 and 42 indicate the cultures were grown at 30 °C or 42 °C. At 42 °C, both mature and precursor ribose-binding protein were overproduced by wild-type and revertant strains and precursor protein was overproduced by the mutant strain. M.W. indicates molecular weight markers. The positions for precursor and mature ribose-binding protein are indicated by P and M, respectively.

at 30 °C or at 42 °C are shown in Fig. 2.

Precursor ribose-binding protein in the revertant strain is accumulated to a greater extent than precursor ribose-binding protein in the wild-type strain. We saw only a little difference in the processing of precursor ribose-binding protein in strains carrying the wild type or revertant gene on the low copy number plasmid pACYC184. Therefore, it seems that the overproduction of protein under lambda P_L promoter control makes a minor defect that is present in the revertant strain more apparent.

Strain SP114, SP115, and SP116 were grown at 42 °C for up to 6 hrs to examine the time course of expression. For all three strains, by 4 hrs after the shift to 42 °C there was no further increase in the amount of precursor or mature ribose-binding protein. (data not shown). Roughly, ribose-binding proteins produced were 30% of the total *E. coli* protein. Thus for purification, cells were harvested 4 hrs after the temperature shift. Purification was done following the procedures described in Materials and Methods.

Purification

The proteins were purified by the procedure described in Materials and Methods. Strains, SP114, SP115, and SP116 were grown, harvested, and subjected to osmotic shock. Following osmotic shock the cells were lysed by treatment with Tris-EDTA-lysozyme and then fractionated with ammonium sulfate precipitation. The material precipitated between 0%-65% ammonium sulfate contained most of the ribose-binding protein. This fraction was dialyzed against 20 mM Tris. Cl pH 7.3 in preparation for DEAE-sephadex column chromatography.

After dialysis, the sample was loaded onto a DEAE-anion exchange column and eluted from the column by a gradient of NaCl from 0.0 M to 0.15 M. Ribose-binding protein was separated from other proteins since it does not bind to the column and comes out in the void volume.

The fractions enriched for ribose-binding proteins obtained from DEAE-anion exchange chromatography were dialyzed against 10 mM Na. Acetate pH 5.0, concentrated and loaded onto a CM-Sephadex column.

Both mature and precursor ribose-binding proteins were eluted at approximately 0.35 M NaCl (data not shown). Fractions enriched in ribose-binding proteins were pooled and concentrated.

For sequencing and structural analysis of the mature protein and precursor protein, it was necessary to separate these two protein species from each other.

It has been shown that by exploiting the difference of pI between mature maltose-binding protein and precursor maltose-binding protein,

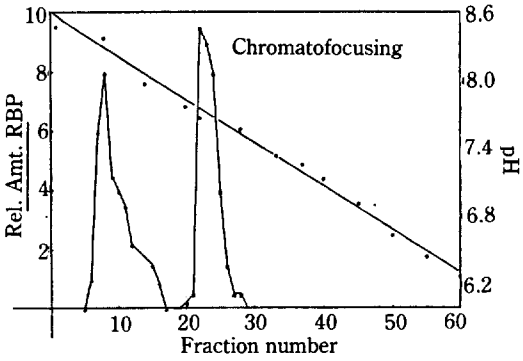

Fig. 3. *Separation of mature and precursor ribose-binding protein using Chromatofocusing column chromatography.*
Pooled fractions enriched in ribose-binding proteins from CM-Sephadex column were dialyzed in 0.025 M Tris.Cl pH 7.3, loaded onto a chromatofocusing column and eluted as described. ●-●, pH gradient developed; ⊙-⊙, elution profile of precursor protein; ⊡-⊡, elution profile of mature protein. Precursor protein was eluted at pH 8.0 and mature protein was eluted at pH 7.5. Rel. Amt. RBP indicates relative amount of ribose-binding protein present in each fraction.

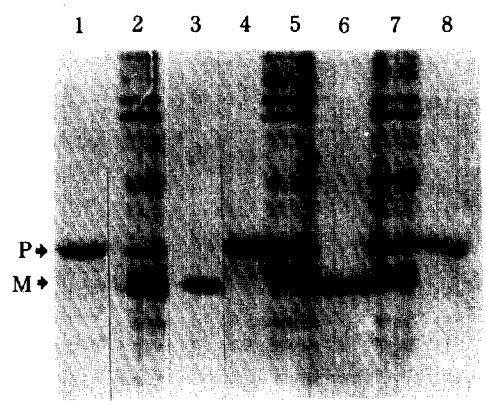

Fig. 4. *Ribose-binding proteins purified to homogeneity.*
Five different species of ribose-binding proteins synthesized from wild-type, mutant, and revertant strains were purified using various column chromatography to homogeneity. Starting materials and purified proteins were compared. The position for precursor and mature ribose-binding protein are indicated by P and M, respectively. Lane 1, wild-type precursor protein. Lane 2, wild-type total protein. Lane 3, wild-type mature protein. Lane 4, mutant precursor protein. Lane 5, mutant total protein. Lane 6, revertant mature protein. Lane 7, revertant total protein. Lane 8, revertant precursor protein.

due to the charged amino acids present in the signal sequence, purification of precursor maltose-binding protein colud be achieved using chromatofocusing colmn chromatography (Ito, 1982).

To separate mature and precursor ribose-binding protein, from each other, pooled fractions enriched in both mature and precursor protein were concentrated, dialyzed against 0.025 M Tris-HCl pH 8.3, and loaded onto a chromatofocusing column. The sample was eluted with Polybuffer 96-acetate pH 6.0 to develop a pH gradient from pH 8.3 to pH 6.0.

The presence of charged amino acids in the signal sequence of precursor affects the pI of the protein and allows separation of the precursor and mature on this column as shown (Fig. 3). The gradient developed was from pH 8.6 to pH 6.3. Precursor protein was eluted at approximately pH 8.0 and mature protein was eluted at approximately pH 7.5. It has been reported that pI of mature ribose-binding protein is about 6.6 using analytical polyacrylamide slab gel electrofocusing (Willis and Furlong, 1974). The discrepancy between these two values might be due to the experimental error in measuring pH of the column fraction. This chromatofocusing step resulted in the purification of mature and precursor protein to homogeneity. The yield for precursor protein was less than 10 % due to the extreme degradation of proteins. The yield for mature protein was more than 90%. The final yields were approximately 1 % for precursor proteins and approximately 10 % for mature proteins. The purity for both species of ribose-binding protein was higher than 99%. The estimation was based on the silver staining and densitometer scanning (data not shown). Fig. 4 shows starting materials and purifed proteins for all five different species of ribosebinding proteins from the wild-type, the mutant, and the revertant strain. Unfortunately, only a small amount of pure wild-type precursor protein was obtained due to low expression initially and extensive degradation during purification. The loss of wild-type precursor might be partly due to the hydrophobicity conferred by

													processing ↓			
	-25								-17		-15		-1	1		3
1 DNA seq. :	ATG	AAC	ATG	AAA	AAA	CTG	GCT	ACC	CTG	GTT	TCC	···············	GCA	AAA	GAC	ACC
2 A.A. seq.d :	MET	ASM	MET	LYS	LYS	LEU	ALA	THR	LEU	VAL	SER	···············	ALA	LYS	ASP	THR
		-24	-23	-22										1	2	3
3 MUTANT p:		ASN	MET	LYS	LYS	LEU	ALA	THR	PRO	VAL	ARG	···············		LYS	ASP	GLU
4 REVERT.p :		ASN	MET	LYS	LYS	LEU	ALA	?	PRO	VAL	PHE	···············		LYS	ASP	GLU

Fig. 5. *Amino acid sequence analyses of purified ribose-binding proteins.*
Line 1: DNA coding for signal sequence of wild-type precursor protein
Line 2: amino acid sequence deduced for signal sequence of wild-type precursor protein
Line 3: amino terminal amino acid sequence determined for mutant precursor protein
Line 4: amino terminal amino acid sequence determined for revertant precursor protein
Arrow indicates the leader peptidase processing site. "•" indicates that the amino acid sequence in that position was not determined.

the signal sequence, resulting in the adherence of proteins to glass surfaces since protein was lost even after it had been purified, eliminating the possibility that the loss was due to digestion by proteases. Thus only mutant precursor and revertant precursor protein were available for amino acid sequenching and conformational analyses.

Amino Acid Sequences of Ribose-binding Proteins

Several amino-terminal amino acid sequences were determined. We verified that the amino acid change in the mutant signal sequence at -17 is proline and in the revertant at -15 is phenylalanine (Fig. 5)

As shown in the amino acid sequence deduced from DNA sequence, there are two potential translation initiation sites for the precursor. Fig. 5 shows that both mutant and revertant precursor were translated from the first potential translation start site. These results imply that wild-type precursor protein is translated at the same site, even though this is not directly shown.

The amino terminal amino acid of both precursors was asparagine instead of methionine. this implies that formyl methionine has been lost while translation continues. One thing that puzzles us now is the presence of arginine at -15 in the mutant signal sequence. We have not been able to find this change in the DNA sequencing. The amino acid at -18 of the revertant signal sequence was not confirmed because of high background in the H.P.L.C. system used for analyzing amino acid sequencing.

One puzzle found by amino acid sequencing was that the amino acid at position 3 in both wild-type and revertant mature protein is glutamine instead of threonine, the amino acid deduced from the wild-type DNA sequence. Since both DNA sequencing of the wild-type gene for strain K12 and mutant gene for strain B showed the deduced amino acid in this position is threonine, this presence of glutamine may be the result of the overproduction of this protein. However, we do not have any clear answer for this finding yet.

DISCUSSION

Cloning of three alleles of *rbsB* under the control of lambda P_L promoter were successfully done. Furthermore, purification of five different species of ribose-binding protein to the homogeneity were accomplished. One thing noticed while ammonium sulfate fractionation was performed was that ribose-binding proteins were pelleted at 0-65% cut instead of being pelleted between 65%-95%. Since it was reported that ribose-binding protein induced in *E. coli* K12 strain by ribose was pelleted between 65%-95%, it seemed that the overproduction caused aggregation. However when cultures were processed for osmotic shock process or Tris-EDTA lysozyme lysis, most of ribose-binding proteins were in supernatant. Thus, it didn't look like ribose-binding

protein accumulates as inclusion bodies, which was usally the case for overproduced proteins. This may be the results of ribose-binding protein being folded fast after synthesis and/or having globular, compact shape.

Unfortunately, we could not purify the wild-type precursor protein not only because the level of initial accumulation of wild-type precursor protein was low but because it was easily lost during purification. This suggests that the leader sequence in the wild type precursor protein could destabilize the protein so that it might be digested by proteases more easily than other precursor proteins. Alternatively, the hydrophobicity of the wild-type signal sequence might cause this protein to adhere to the surface of glass tubes resulting in loss during purification.

Several modifications may improve a purification of wild-type precursor such as keeping the sample temperature at 30 °C after lysing cells. This may induce folding rapidly and render the protein resistanct to proteolysis. Alternatively the sample might be kept cool with more care, thus maintaining the native conformation of the protein while minimizing degradation by proteases. Also, use of affinity chromatography made of antibody against ribose-binding protein could reduce the purification steps, thereby minimizing degradation.

적 요

신호배열 돌연변이인 *rbsB* 103는 전구체 리보스 결합단백질을 세포질내에 축적시키고, *rbsB* 106 복귀유전자는 이 전구체를 숙성 가능하게 하여 페리플라슴으로 수송되게 한다(Iida *et al.*, 1985, Park, *et al.*, 1988). 본고에서는 *rbsB* 유전자의 세 allele, *rbsB*, *rbsB* 103와 *rbsB* 106를 이들이 코딩하는 단백질을 대량생산 하고자 람다 P_L 프로모터 조절하에 클론하고 나아가서 다섯종의 단백질을 순수정제하였다. 전구체 단백질의 pI는 8.0, 숙성단백질의 pI는 7.5임을 밝혔다. 순수정제된 단백질의 아미노 말단의 아미노산 배열을 결정하여 DNA 염기서열로부터 밝혀진 아미노산 변화를 확인하였다.

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(Received July 5, 1988)