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Processing of preproglycinin expressed from cDNA-encoding $A_{1a}B_{1b}$ subunit in *Saccharomyces cerevisiae*

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The cDNA sequence encoding the glycinin $A_{1a}B_{1b}$ subunit was placed under control of the repressible-acid phosphatase promoter of the yeast *Saccharomyces cerevisiae* in an expression plasmid. Yeast transformants containing these plasmids synthesized $A_{1a}B_{1b}$ protein. The signal sequence of the $A_{1a}B_{1b}$ protein was cleaved at the right processing site and the $A_{1a}B_{1b}$ protein was not glycosylated in yeast in the same manner as in soybean.

Glycinin; Preproglycinin; Signal sequence; Processing; (Yeast)

1. INTRODUCTION

Glycinin, one of the predominant storage proteins of soybean (*Glycine max* L.), is composed of six subunits, each of which consists of an acidic and a basic polypeptide that are linked by a disulfide bond [1–4]. A single polypeptide precursor consisting of covalently linked acidic and basic polypeptides with a signal sequence is initially synthesized. The signal peptide is removed cotranslationally in the endoplasmic reticulum [5]. The resultant proglycinin subunits assemble into trimers of about 8 S [6,7]. The proglycinin subunits move into the protein bodies and are cleaved to form the acidic and basic polypeptides and assemble into hexamers of about 12 S [6,7].

We have demonstrated that the polymorphism of cDNAs encoding glycinin subunits occurs among cultivars [8,9], and that polymorphic $A_{1a}B_{1b}$ cDNAs are distributed in one cultivar [8]. To clarify the relationship between the diversity of expressed products and protein functions, it is necessary to investigate the expression of the cDNAs in microorganisms. Here, we examined the expression of the $A_{1a}B_{1b}$ cDNA employing repressible-acid phosphatase promoter (PHO5) in yeast.

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2. MATERIALS AND METHODS

2.1. *Strains, media and plasmids*

Yeast strain AH22 *(a leu2 his4 can1 cir$^+$)* and a yeast-*E. coli* shuttle vector pAM82 with a PH05 promoter were the gifts of Dr K. Matsubara [10]. Plasmid pGST4-2-11-10 is a derivative of Okayama-Berg vector [11] carrying the cDNA encoding the $A_{1a}B_{1b}$ subunit [8]. Burkholder minimal medium [12] fortified with histidine (20 μg/ml) was used for preparation of high-P_i medium (1.5 mg KH_2PO_4/ml) or low-P_i medium (1.5 mg KCl/ml, in place of P_i).

2.2. *Construction of the expression plasmid pAMA$_{1a}$B$_{1b}$*

Expression plasmid $pAMA_{1a}B_{1b}$ was constructed by inserting a cDNA region of pGST4-2-11-10 into the filled-in *Xho*I site of pAM82 as shown in fig.1. Briefly, in order to eliminate the oligo(dG)tail from the 5′-noncoding region, the Cfr13I-*Acc*I and *Acc*I-*Sca*I fragments of pGST4-2-11-10 were prepared by 4.2% polyacrylamide gel electrophoresis and inserted into the filled-in *Xho*I site of pAM82 which is located downstream from the PH05 promotor [10].

2.3. *Transformation of yeast cells*

Transformation was performed according to Ito et al. [13]. Leu$^+$ transformants were selected on SD medium containing histidine (20 μg/ml) and 2% agar.

2.4. *Expression and detection of glycinin A$_{1a}$B$_{1b}$ subunit*

AH22 cells harboring $pAMA_{1a}B_{1b}$ were grown in 10 ml high-P_i Burkholder minimal medium supplemented with histidine (20

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μg/ml) at 30°C. At $A_{610}=0.3$, yeast cells were collected and suspended in 10 ml low-P_i minimal medium for induction. Control samples were treated similarly, except that high-P_i minimal medium was used. At $A_{610}=1.0$, yeast cells were centrifuged. The resultant cells were lysed by addition of NaOH/2-mercaptoethanol and proteins were applied on SDS-polyacrylamide gels as described [14,15]. The proteins separated on SDS gels were transferred to nitrocellulose filters and detected by the immunological method in [9], employing glycinin antibodies purified on a glycinin-Sepharose column [16].

2.5. *Purification of $A_{1a}B_{1b}$ protein expressed in yeast*

Cultures (1000 ml) induced as described in section 2.4 were harvested by centrifugation and the cells were homogenized on a Brown homogenizer (B. Brown, type 2876, Apparatebau Melsungen, FRG) with glass beads in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100. Extracts (10 ml) were clarified by centrifugation, and fractionated by addition of ammonium sulfate into 30 and 60% saturated fractions and the supernatant. The 30% saturated fraction containing the expressed $A_{1a}B_{1b}$ protein was dissolved in and dialysed against 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl (coupling buffer). The dialysate was applied on a glycinin antibody-Sepharose column, which was equilibrated with coupling buffer. After being washed thoroughly with coupling buffer, the expressed $A_{1a}B_{1b}$ protein was eluted with 0.01 M potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 8 M urea.

2.6. *Determination of N-terminal amino acid sequence*

The N-terminal amino acid sequence was determined using a gas-phase protein sequencer (Applied Biosystems, model 477A) after the protein sample (12 μg) separated on SDS gels was blotted onto a PVDF membrane (Millipore) according to Matsudaira [17].

2.7. *Analysis of glycosylation*

The $A_{1a}B_{1b}$ protein (10 μg) purified from yeast was applied onto SDS gels for electrotransfer to nitrocellulose filters, and then analysed with peroxidase-conjugated Con A (Seikagaku Kogyo, Japan) as recommended by the manufacturer.

3. RESULTS

3.1. *Expression of glycinin $A_{1a}B_{1b}$ cDNA in yeast*

A cDNA fragment encoding glycinin $A_{1a}B_{1b}$ subunit was linked to the promoter region of the *Saccharomyces cerevisiae* repressible-acid phosphatase gene (PH05) in a shuttle vector pAM82 as shown in fig.1. An expression plasmid having the cDNA insert in the proper orientation for expression was identified by restriction-endonuclease analysis. The expression plasmid $pAMA_{1a}B_{1b}$ propagated in *E. coli* HB101 was transformed into a yeast recipient strain, AH22, selecting for Leu^+ colonies.

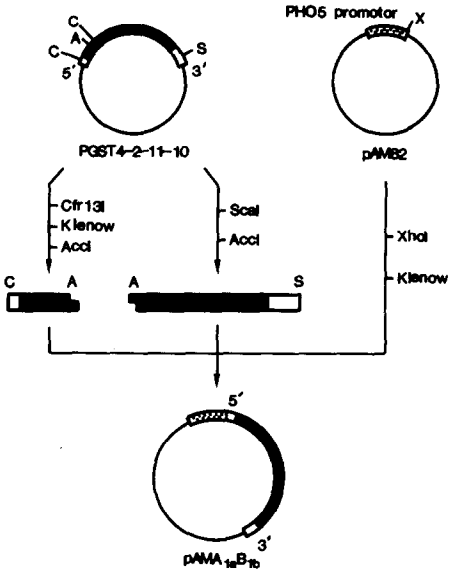

Fig.1. Construction of expression plasmid for glycinin $A_{1a}B_{1b}$ subunit. (Filled sections) Preproglycinin coding sequence, (unfilled) 3'- and 5'-noncoding regions of $A_{1a}B_{1b}$ cDNA, (stippled) PH05 promoter. A, C, S, and X denote the *Acc*I, Cfr131, *Sca*I and *Xho*I sites.

The Leu^+ cells were grown in liquid medium and then induced in phosphate-free medium. After cultivation for induction, the soluble protein extracts were applied to SDS-polyacrylamide gels. A

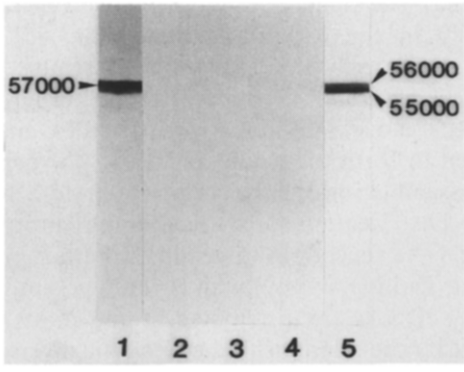

Fig.2. Immunodetection of soybean glycinin $A_{1a}B_{1b}$ protein in yeast. Samples were applied to 11% SDS-polyacrylamide gels in the presence of 0.2 M 2-mercaptoethanol. After transfer to nitrocellulose filters, the filters were treated with glycinin antibodies. Lanes: 1, induced AH22 harboring $pAMA_{1a}B_{1b}$; 2, AH22 harboring pAM82 alone; 3, non-induced AH22 harboring $pAMA_{1a}B_{1b}$; 4, media of induced AH22 harboring $pAMA_{1a}B_{1b}$; 5, glycinin purified from soybean in the absence of 2-mercaptoethanol.

protein of $M_r \sim 57\,000$ produced in yeast harboring expression plasmid was recognized by the glycinin antibodies (fig.2, lane 1). The size of this protein is close to the apparent M_r of 55000–56000 of mature glycinin subunits (lane 5). No protein from the control cells harboring pAM82 alone (lane 2) and from non-induced cells (lane 3) was detected by the glycinin antibodies. This suggests that glycinin $A_{1a}B_{1b}$ subunit protein was synthesized under the control of the PH05 promotor in yeast. Densitometric analysis of the proteins on SDS gels indicated that the yeast-synthesized glycinin composed approx. 0.3% of the total soluble proteins. The $A_{1a}B_{1b}$ protein was not detected in the media (lane 4), indicating that the $A_{1a}B_{1b}$ protein expressed in yeast was not secreted.

3.2. *Determination of the N-terminal amino acid sequence of the $A_{1a}B_{1b}$ protein expressed in yeast*

The expressed protein was purified and then the N-terminal amino acid sequence was analysed. The expressed protein was predominantly precipitated at 30% saturation of ammonium sulfate (fig.3A, lane 2). This phenomenon was visualized with Coomassie brilliant blue stain (fig. 3B, lane 2). The 30% saturated fraction was applied to the glycinin antibody-Sepharose column and eluted with urea. The expressed protein was purified to higher than 90% purity (fig.3C). The protein separated on SDS gels was transferred to PVDF membranes and sequenced from the N-terminal according to Matsudaira [17]. The sequence was determined to be: Phe-Ser-Ser-(X)-Glu-Gln-Pro-Gln-Gln-Asn-Glu-. This is consistent with the N-terminal sequence of mature A_{1a} polypeptide predicted from the nucleotide sequence of $A_{1a}B_{1b}$ cDNA [8]. Therefore, the expressed protein in yeast harboring $pAMA_{1a}B_{1b}$ is identified with $A_{1a}B_{1b}$ protein. It is likely that the plant signal sequence is correctly recognized and cleaved at the same processing site as in soybean, by the yeast processing system.

3.3. *Analysis of glycosylation*

Glycinin is not a glycoprotein. However, the nucleotide sequence of $A_{1a}B_{1b}$ cDNA revealed that there is one potential site for *N*-glycosylation: the asparagine residue at position 418. After electrotransfer of the protein separated on the SDS gels to nitrocellulose filters, the blots were allowed

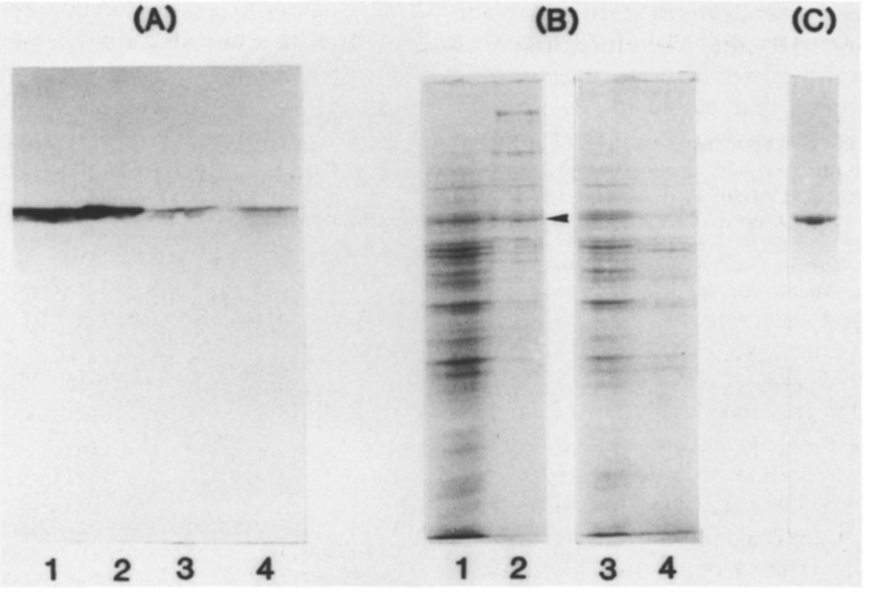

Fig.3. (A) Ammonium sulfate fractionation of the extract from the yeast harboring $pAMA_{1a}B_{1b}$. The extract was fractionated by the addition of ammonium sulfate. Each fraction was applied to 11% SDS gels and detected by immunoblotting. Lanes: 1, extract; 2, 30% saturated fraction; 3, 60% saturated fraction; 4, supernatant of 60% saturated fraction. (B) SDS gels identical to those in A were stained with Coomassie brilliant blue. Arrow indicates the expressed protein. (C) SDS gel of the $A_{1a}B_{1b}$ protein purified by the glycinin antibody-Sepharose column chromatography of the 30% saturated fraction. The gel was stained with Coomassie brilliant blue.

to interact with peroxidase-conjugated Con A. The protein did not interact with Con A, suggesting that the $A_{1a}B_{1b}$ protein expressed in the yeast is not *N*-glycosylated.

4. DISCUSSION

Here, we have demonstrated (i) that cDNA encoding soybean glycinin $A_{1a}B_{1b}$ subunit can be expressed efficiently in yeast cells under control of the yeast acid phosphatase promoter, (ii) that the signal sequence of the expressed preproglycinin is cleaved at the same processing site as in soybean by the yeast processing system, and (iii) that the proglycinin is not *N*-glycosylated.

In the course of this study, Fukazawa et al. [18] reported that cDNAs encoding $A_{1a}B_{1b}$ and A_2B_{1a} subunit precursors are expressed in *E. coli,* and that the expressed products have molecular masses in agreement with those of the in vitro translation products directed by their mRNAs. This suggests that the signal sequences are not processed in *E. coli.* Dickinson et al. [19] suggested that the folding of preproglycinin was disrupted by the presence of a hydrophobic signal sequence. However, the signal sequence of the preproglycinin expressed in yeast was correctly recognized and processed at the correct site. Therefore, it may be concluded that the expression system employing yeast is superior to that employing *E. coli* for studying the structures and functions of engineered polymorphic glycinins.

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