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Stability and detection of recombinant pre-pro-concanavalin A after cytoplasmic expression in *Escherichia coli*

Wang Min and D. Hugh Jones

Molecular Biology Research Group, School of Biological Sciences, University College of Swansea, Swansea, SA2 8PP, Wales, UK

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The cDNA for pre-pro-concanavalin A (pre-pro-Con A) from *Canavalia ensiformis* was used to construct two cytoplasmic expression vectors: pKconA (no transcription terminator) and pTKconA (containing the *cry* transcription terminator). The latter produced 2- to 3-fold greater amounts of pre-pro-Con A. This product containing the plant signal can be detected by Western blotting only after electrophoretic transfer in the presence of SDS, indicating reduced solubility. The signal is not removed and pre-pro-Con A is clearly stable after expression in *E. coli* JM109. The protein is not cleaved and ligated as in the plant, in contrast to a recent report.

Concanavalin A; Legume lectin; Post-translational processing; Recombinant protein stability; Transcription terminator; Western blotting; *Canavalia ensiformis*; *Escherichia coli*

1. INTRODUCTION

Concanavalin A (Con A) is a lectin [1] from *Canavalia* species for which a highly unusual mechanism of post-translational processing has been demonstrated [2–4]. In developing seeds, the plant signal peptide of pre-pro-Con A is removed during secretion into the endoplasmic reticulum. The precursor (pro-Con A) is glycosylated and then packed into protein bodies in which it is processed through a complex series of events involving deglycosylation, peptide cleavage and religation to yield mature active lectin [5].

It has been reported [6] that pre-pro-Con A from *Canavalia gladiata* expressed in *Escherichia coli* undergoes a peptide cleavage and ligation in the same way as that synthesized during seed maturation. In contrast to this finding, we describe here the expression of pre-pro-Con A which is stable and is not processed further in *E. coli*.

2. MATERIALS AND METHODS

2.1. *Materials*

The cDNA for pre-pro-Con A [2] was a generous gift from D.M. Carrington and D.E. Hanke: the coding sequence was provided as an insert in the *Bam*HI site of pGEM-1 (Promega) to give pGEMconA. Plasmid pR70, a kind gift from L.H. Guo, Shanghai Institute of Cell Biology, P.R. China, carries 87 bp of the *cry* [7] transcription terminator sequence in the *Xba*I and *Bam*HI sites of pWR13 [8]. pKK233-2 [9] was obtained from Pharmacia.

Abbreviations: Con A, concanavalin A; IPTG, isopropyl-1-thio-β-D-galactopyranoside; RBS, ribosome binding site.

Correspondence address: D.H. Jones, Molecular Biology Research Group, School of Biological Sciences, University College of Swansea, Singleton Park, Swansea, SA2 8PP, Wales, UK. Fax: (44) (792) 295447.

2.2. *Construction of expression vectors (Fig. 1)*

The *Sal*I–*Nco*I fragment (0.8 kb) containing *trc* promoter and ribosome binding site (RBS) from pKK233-2 was cloned to the *Sal*I and *Nco*I sites of pGEMconA to form the plasmid pKconA. This construct was digested with *Eco*RI and *Bam*HI and the 1.9 kb fragment (containing the 1.1 kb cDNA sequence for pre-pro-Con A) was inserted into the *Eco*RI and *Bam*HI sites of pR70 to form the plasmid pTKconA. Recombinant DNA manipulations were carried out according to [10].

2.3. *Bacterial growth and induction conditions*

E. coli strain JM109 harbouring either plasmid pR70, pKconA or pTKconA was grown in M9 medium [10], supplemented with 0.5% (w/v) CAS-amino acids, glucose (4 mg/ml), thiamine (2 μg/ml) and ampicillin (100 μg/ml) at 37°C, 250 rpm, to an A_{600} of 0.5–0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation was continued for a further 5–6 h.

2.4. *Detection of expressed products*

Cells were collected by centrifugation, boiled in SDS sample buffer, and total proteins were analysed by SDS-electrophoresis on 12.5% polyacrylamide gels [11]. Expression of recombinant products was detected by Western blotting using a Bio-Rad Immuno-blot assay kit (alkaline phosphatase) and rabbit anti-Con A serum (Sigma). Blotting membranes were from Amersham (Hybond-C, Super). Two different transfer buffers were used [12]. Transfer Buffer 1 contained SDS (50 mM Tris base, 380 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) and Transfer Buffer 2 did not contain SDS (25 mM Tris base, 190 mM glycine, 20% (v/v) methanol). Electrophoretic transfer was performed at 100 V for 1 h with external cooling. A current of 550 mA was recorded for Transfer Buffer 1, and 360 mA for Transfer Buffer 2.

3. RESULTS AND DISCUSSION

Starting from plasmids pKK233-2 and pGEM-conA, two expression vectors pKconA and pTKconA were constructed (Fig. 1). The correct constructs were con-

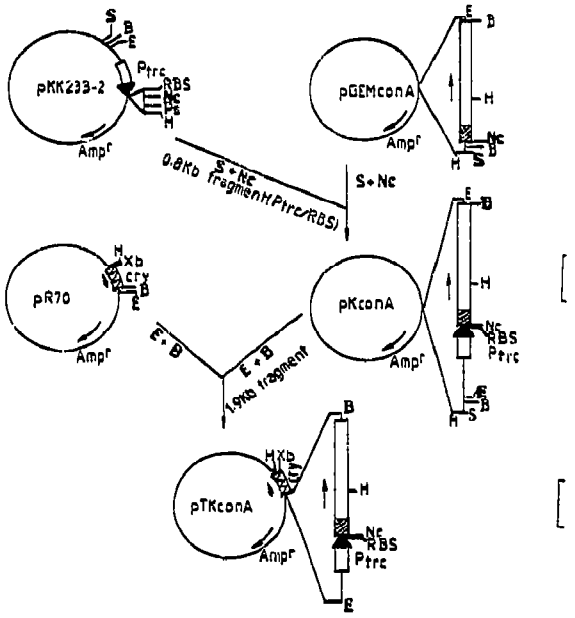

Fig. 1. Construction of pre-pro-Con A expression vectors pKconA and pTKconA. ⟹, *trc* promoter (P_{trc}); ▨, plant signal sequence; ▨, *cry* terminator; ▭, pro-Con A coding sequence. The *Nco*I site, C↓CATGG in which the initiation codon is underlined, was used to excise the coding sequence corresponding to the N-terminus. Bold lettering in the figure indicates the restriction sites used at each construction step. Restriction fragments obtained as confirmation of these constructs are shown bracketed on the right hand side. The restriction sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Nc, *Nco*I; Ps, *Pst*I; S, *Sal*I; Xb, *Xba*I.

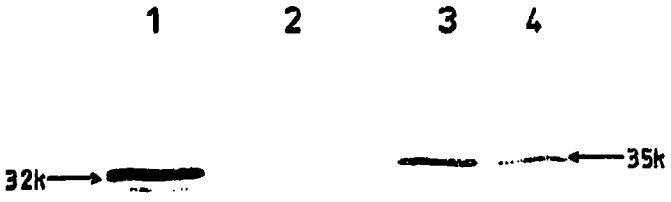

Fig. 2. The detection of recombinant products by Western blotting (Transfer Buffer I containing SDS was used). Lane 1, purified rec-pro-Con A from W620*recA*(pINpro) [13]. Lanes 2–4 show blots of total protein extracts from the following cultures: lane 2, JM109(pR70); lane 3, JM109(pTKconA); lane 4, JM109(pKconA). (Note: mature Con A (Fig. 3, lane 4), M_r = 25,600 (calculated from amino acid sequence [16] plus metal ions) to 26,500 (generally reported [1]) migrates anomalously to a position corresponding to 30,000 on SDS-PAGE [3,4]. Rec-pro-Con A (Fig. 2, lane 1) predicted M_r = 28,200 from translated DNA sequence migrates to approximate position 32,000 [13]. Pre-pro-Con A (Fig. 2, lanes 3 and 4, and Fig. 3A,B, lane 1) predicted M_r = 31,500 from translated DNA sequence migrates to approximate position 35,000.)

firmed by digestions with appropriate enzymes. A double digest with *Nco*I and *Sal*I of pKconA gave a 0.8 kb fragment. A *Bam*HI digest of pKconA gave a 1.9 kb fragment, whereas pGEM-conA yielded a 1.1 kb fragment after *Bam*HI digestion. A double digest with *Eco*RI and *Bam*HI of pTKconA gave a 1.9 kb fragment, and a double digest with *Eco*RI and *Nco*I of pTKconA yielded a 0.8 kb fragment.

The expression vector pKK233-2 is often useful for expression of foreign genes in *E. coli*, as the *Nco*I recognition sequence commonly occurs at the initiation codon of eukaryotic genes [9]. The *Nco*I site in the pre-pro-Con A coding sequence cloned in pGEMconA is shown in Fig. 1. The resultant plasmids pKconA and pTKconA both carry the *trc* promoter and RBS of pKK233-2, followed by the ATG initiation codon and entire cDNA sequence for pre-pro-Con A. In addition, pTKconA contains the synthetic *cry* transcription terminator [7] downstream of the pre-pro-Con A insert.

In other work [13], we used the expression vector pIN-III-ompA and showed that non-glycosylated pro-Con A (rec-pro-Con A) expressed in *E. coli* folds in vivo and in vitro to a stable form which is active without further processing. In that system, a bacterial outer membrane protein (*ompA*) signal peptide was correctly cleaved in the periplasm to produce pro-Con A with the expected N-terminal sequence.

As shown by Western blotting (Fig. 2), cells harbouring plasmid pKconA or pTKconA give only one band of higher molecular weight (M_r) than rec-pro-Con A (which was previously purified from *E. coli* W620*recA*(pINpro) [13]). This indicates that the plant signal peptide of pre-pro-Con A is not removed by bacterial signal peptidase. The control culture harbouring pR70 does not give a corresponding band. No immunologically related bands of lower M_r are seen (Fig. 2, lanes 3 and 4) and pre-pro-Con A is clearly expressed in a stable form. Cultures of JM109(pTKconA) express 0.5–1.0 mg/l of pre-pro-Con A (Fig. 2, lane 3). It has been reported [6] that pre-pro-Con A expressed in *E. coli* undergoes a peptide cleavage and ligation in the same way as that synthesized during plant seed development [4] to produce mature Con A. Neither pre-pro-Con A nor pro-Con A were detected by these authors [6]. This contrasts with our results (Fig. 2) where pre-pro-Con A is found as an intact molecule retaining the original signal peptide. In our laboratory, it appears that both pre-pro-Con A [this paper] and pro-Con A [13] are stable after expression in *E. coli* and no further processing akin to that in the jack bean [4,5] can be detected.

The *cry* terminator has been shown to increase the half-life of mRNAs and so enhance the expression of the upstream genes [7]. This enhancement was independent of the insertional orientation of a distal *cry* terminator [7]. In this work, we inserted the *cry* terminator downstream of the pre-pro-Con A sequence and showed that the culture of JM109(pTKconA) (Fig. 2, lane 3) accumulated 2- to 3-fold greater amounts of pre-pro-Con A

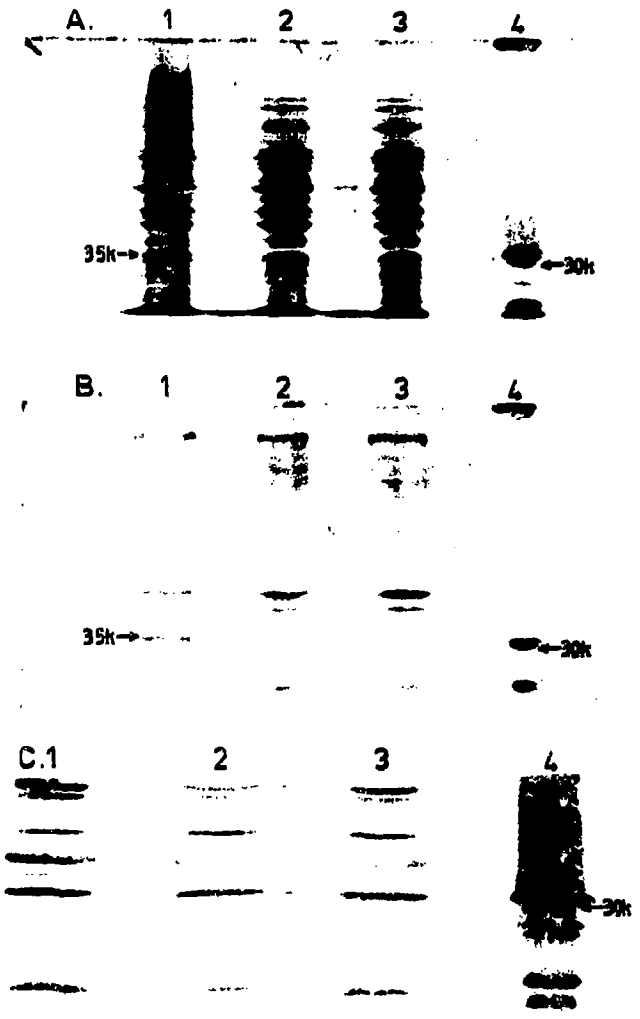

Fig. 3. Inefficient electrophoretic transfer of recombinant products in Transfer Buffer 2 (without SDS). A: Gel before transfer; B: Gel after transfer. Both gels (A) and (B) were stained with Coomassie brilliant blue R. C: Corresponding Western blot. An extended incubation with both first and second antibody solutions was used to maximise the sensitivity of detection of recombinant products. This resulted in a high background and many non-specific bands in this blot as compared to that in Fig. 2. Lanes 1–3 show total protein extracts from the following cultures: lane 1, JM109(pTKconA; lane 2, JM109(pKconA); lane 3, JM109(pR70); lane 4, standard mature Con A (Sigma, Type IV). (M_r arrows: see note at end of legend to Fig. 2.)

during 5–6 h induction than did the culture of JM109(pKconA) (Fig. 2, lane 4). This agrees with the observation that the expression of penicillin G acylase was enhanced 2- to 3-fold by the *cry* terminator (L.H. Guo, pers. comm. and [8]). Moreover, the pTKconA construct is itself more stable in *E. coli* JM109 than the pKconA construct when cultures are induced for longer periods. Stability was assessed by recovering plasmids after overnight induction and then estimating the amounts present by their subsequent ability to re-transform *E. coli* JM109. The *cry* terminator had no effect on plasmid recovery after growth in the absence of inducer, but after 14–16 h exposure to 1 mM IPTG the levels of pKconA declined to about one-fifth those of pTKconA, and the yield of pre-pro-Con A from pKconA thus fell below detection limits.

In conventional electrophoretic transfer buffer without SDS (Transfer Buffer 2), pre-pro-Con A remained in the SDS-polyacrylamide gel, whereas other protein bands were transferred to the blotting membrane (Fig. 3B,C). Although pre-pro-Con A can be seen on an original Coomassie-stained SDS-polyacrylamide gel (Fig. 3A) and on the gel stained after electroblotting (Fig. 3B), no corresponding band was observed on the Western blot (Fig. 3C). In related work [13], we noted that pre(*ompA*)-pro-Con A was less soluble than pro-Con A since pre(*ompA*)-pro-Con A largely remained in pelleted cell debris or precipitated during purification. An increase in the length from 21 residues (*ompA* signal) to 29 residues (original plant signal) would be expected to increase the hydrophobicity and tendency to aggregate, and so further decrease solubility of the recombinant product. Addition of SDS (0.1%, w/v) to the transfer buffer (Fig. 2) (as suggested in the Instruction Manual for the Bio-Rad Trans-blot Electrophoretic Transfer Cell) overcomes detection problems (Fig. 3).

In conclusion, the work of Yamauchi and Minamikawa [6] directly implies the occurrence in *E. coli* of a plant-seed-like endopeptidase which cleaves only on the C-side of asparagine residues [4,14,15]. We can find no evidence for the presence of such a bacterial enzyme either in cytoplasm (this paper) or periplasm [13].

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