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The reaction specificities of the pea and a cyanobacterial thylakoid processing peptidase are similar but not identical

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The thylakoid processing peptidase from the cyanobacterium *Phormidium laminosum* has been extracted from thylakoid membranes by solubilization with Triton X-100. Its reaction specificity has been compared with the analogous pea peptidase by processing in vitro of radiolabelled wheat and *P. laminosum* thylakoid lumenal precursor polypeptides. The cyanobacterial polypeptide is processed to the mature size through an intermediate by the *P. laminosum* peptidase, but to a polypeptide that has a slightly greater apparent molecular weight than the intermediate by the pea peptidase. Both peptidases correctly process the wheat polypeptide. This suggests that the reaction specificities of the two peptidases are similar, but not identical.

Thylakoid lumenal protein; Leader sequence; Processing peptidase; Cyanobacteria; Pea

1. INTRODUCTION

In plants, nuclear-encoded thylakoid lumenal polypeptides are synthesized in the cytoplasm as precursors with an N-terminal extension that can be divided into two functionally distinct domains [1,2]. The N-terminal domain directs import of the polypeptide into the chloroplast stroma where it is removed by a stromal peptidase [2,3]. The C-terminal, thylakoid-transfer domain is structurally similar to prokaryotic and eukaryotic leader sequences that target polypeptides across the plasma membrane and into the lumen of the endoplasmic reticulum, respectively. These have a positively charged N-terminus followed by a long hydrophobic stretch and a peptidase cleavage site. A membrane-bound peptidase removes the thylakoid-transfer domain during or shortly after import of the polypeptide into the lumen [4].

With few exceptions [5,6], no primary sequence homology has been observed between leader sequences from any sources. It is thought, therefore, that it is the types of amino acids at cleavage sites that are important for recognition by leader sequence peptidases. Consistent with this, the pea thylakoid peptidase correctly processes precursors from wheat and *E. coli* that have different primary sequences at the cleavage sites [7]. An analysis of the residues at positions −3 and −1 relative to the cleavage sites shows that they have small, uncharged side chains [8,9]. It may be that it is these features rather than the primary sequences that are recognized by the peptidases. However, it is not clear how flexible the reaction specificity of a particular enzyme is and whether a peptidase will process all precursors with such features at the cleavage site.

Cyanobacteria are prokaryotes that, like plants, have thylakoid membranes which contain polypeptide complexes that participate in oxygenic photosynthesis. Since the thylakoid membranes are not contained within chloroplasts, the leader sequences of lumenal polypeptides lack the chloroplast-import domain although they are structurally similar to the thylakoid-transfer domain of plants [6,10,11]. In view of this similarity, we have partially purified a thylakoid processing peptidase from a cyanobacterium and used it to see whether cyanobacterial processing enzymes will process higher plant precursors and vise versa.

2. MATERIALS AND METHODS

The polypeptides used in this study were the precursors of the extrinsic 23 kDa polypeptide of the 'oxygen-evolving complex' from wheat [12] and the 9 kDa polypeptide that is involved in the oxygen-evolving activity of *P. laminosum* [6,13–15].

2.1. *Preparation of radiolabelled precursor polypeptides*

A 1.45 kbp *Bgl*II fragment of *P. laminosum* genomic DNA containing the gene for the 9 kDa polypeptide [6] was ligated into the *Bam*HI restriction site of the expression vector pGEM-2 (Promega Corporation, London, UK) and the construct containing the gene under the control of the SP6 promoter was used for transcription experiments in vitro. Capped mRNA transcripts were obtained using SP6 RNA polymerase (Promega) and radiolabelled precursor 9 kDa polypeptide was obtained by translating them in vitro using wheat-germ extract (Promega) in the presence of [^{35}S]methionine (13 mCi/ml, spec. act. 1300 Ci/mmol, Amersham International, UK),

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(a) MKRLVGVLMILGLMLTSWGLLGSPQTAIAASLSPLSFNPSPVLAE ▼

(b) VTSRRAALSLLAGAAAIAVKVSPAAAA

Fig. 1. Amino acid sequence of (a) the leader sequence of the *P. laminosum* 9 kDa polypeptide and (b) the thylakoid-transfer domain of the wheat 23 kDa polypeptide. The mature processing sites are indicated by a black triangle.

according to manufacturer's instructions. Radiolabelled precursor 23 kDa polypeptide was obtained as described in [7].

2.2. *Preparation of thylakoid processing peptidases*

P. laminosum was grown and thylakoid membranes prepared as described in [16]. Thylakoid processing peptidase was extracted from *P. laminosum* thylakoid membranes using Triton X-100 (Sigma) as described in [17] except that membranes were used at a concentration of 0.75 mg/ml Chl*a*. The peptidase from pea was prepared as described in [17].

2.3. *Processing of precursor polypeptides*

In processing experiments with the pea peptidase, 16 μl of translation mixture were incubated with 120 μl of pea thylakoid extract at 27°C and 17μl of this was removed at the times indicated in the figures. When the *P. laminosum* peptidase was used, the extract was diluted 10 × (with extract buffer) and 75 μl of this was added to 5 μl of translation mixture. This was incubated at 27°C and 16 μl samples were removed at the times indicated in the figures. As a control, 1 μl of translation mixture was incubated at 27°C with 15 μl of the Triton X-100 extract buffer. After incubation, the processing was immediately stopped by adding 15 μl solubilizing buffer (8% w/v sodium dodecyl sulphate, 20% v/v glycerol, 0.01% w/v Bromophenol blue, 0.2 M Tris-HCl, pH 6.8) and 2 μl β-mercaptoethanol and kept on dry ice before analysis by SDS-PAGE. Samples were thawed, heated at 100°C for 3 min and analysed by SDS-PAGE using 12–23% w/v polyacrylamide slab gels [13]. After electrophoresis gels were soaked in 'Amplify' (Amersham International) and radiolabelled polypeptides visualized by autoradiography.

3. RESULTS

3.1. *Structure of the thylakoid targeting sequences*

Fig. 1 shows the leader sequence and thylakoid-transfer domain of the 9 and 23 kDa polypeptides, respectively [6] (C. Robinson unpublished). The peptidase cleavage sites have been confirmed in both cases by amino acid sequencing of the mature polypeptides [6] (C. Robinson unpublished). Both targeting sequences exhibit the characteristic structure of leader sequences and cleavage sites of polypeptides that are targeted across single membranes, although the hydro-

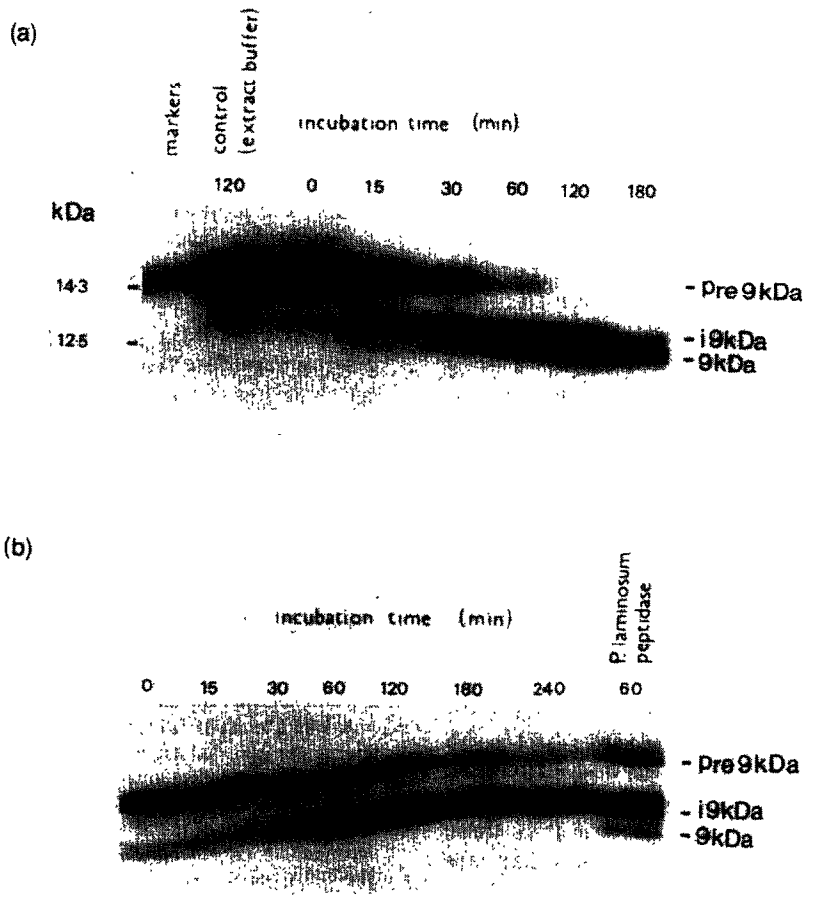

Fig. 2. Time-course of processing in vitro of the radiolabelled precursor *P. laminosum* 9 kDa polypeptide by (a) the *P. laminosum* and (b) pea thylakoid processing peptidases. Pre, precursor; i, intermediate.

phobic domain of the 9 kDa polypeptide is approximately twice the size of the corresponding region in the 23 kDa polypeptide. There is no significant primary sequence homology throughout the length of the two sequences, including the cleavage sites.

3.2. *Processing of the 23 and 9 kDa precursor polypeptides*

Transcription and translation in vitro indicate that 9 kDa polypeptide is initially synthesized as a precursur of 15.5 kDa. This is consistent with the predicted molecular mass of 15.4 kDa from the derived amino acid sequence [6]. The molecular mass of the mature 9 kDa polypeptide was originally estimated by SDS-PAGE [13] but the derived amino acid sequence indicates that the polypeptide will have a molecular mass of 11.3 kDa.

A time-course of processing of the precursor 9 kDa polypeptide by the *P. laminosum* peptidase extract is shown in Fig. 2a. The band corresponding to the mature polypeptide was established by running precursor processing products along side total PSII polypeptides (results not shown). It is apparent that the polypeptide is efficiently processed to the mature size through an intermediate. Using the molecular weights obtained from the derived amino acid sequences, the intermediate has an apparent molecular mass of approximately 12.6 kDa. This would be consistent with intermediate processing within the region of Ala-16 to Ser-14. In some experiments, and after processing with high concentrations of the peptidase for a prolonged period, there was general degradation of the mature polypeptide. There is at least one other peptidase present in *P. laminosum* thylakoids (TPW, unpublished) which could account for this observation. Alternatively, a peptidase in the wheatgerm extract may be responsible.

The pea peptidase has previously been shown to process the wheat precursor 23 kDa polypeptide to the mature size, with no evidence for a processing intermediate [7]. Fig. 3 shows that the precursor 23 kDa polypeptide is also rapidly and completely processed to the mature size by the *P. laminosum* leader sequence peptidase extract. In time-course experiments no evidence could be found for a processing intermediate during processing with either the pea or *P. laminosum* peptidases (results not shown). In the complementary experiment, when the precursor 9 kDa polypeptide is incubated with the pea peptidase the precursor is processed to a polypeptide that has an electrophoretic mobility that is slightly slower than that of the intermediate observed during processing with the *P. laminosum* peptidase (Fig. 2b). Further processing to give the intermediate or mature polypeptide is not observed (Fig. 2b) even after prolonged incubation with an increased volume of peptidase (results not shown). However, the possibility that this would occur under different reaction conditions can not be excluded.

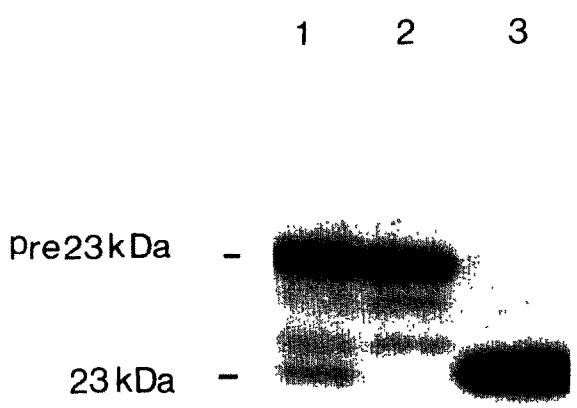

Fig. 3. Processing in vitro of the radiolabelled precursor wheat 23 kDa polypeptide by the pea peptidase (track 1, incubated for 120 min) and the *P. laminosum* peptidase (track 3, incubated for 15 min). Track 2 is unprocessed precursor polypeptide. Symbols as in Fig. 2.

4. DISCUSSION

In this study we have shown that the thylakoid processing peptidase from the cyanobacterium *P. laminosum* can be extracted from thylakoid membranes in an active state using the detergent Triton X-100. The *P. laminosum* precursor 9 kDa polypeptide is a good substrate for the peptidase; it is efficiently processed to the mature size, probably through an intermediate. In all cases tested so far, the thylakoid-transfer domain of lumenal polypeptides are removed by the thylakoid processing peptidase in a single step. In at least one case, however, a chloroplast-imported stromal polypeptide is processed by the stromal peptidase in two stages [18,19]. Interestingly, when a prokaryotic signal sequence cleavage site prediction algorithm [20] is applied to the leader sequence of the 9 kDa polypeptide two potential processing sites are identified. The correct cleavage site scores 3.232 but there is a second site, between Ala-16 and Ala-15, which has a higher score of 4.095. This site is within the region that is predicted by the molecular weight of the intermediate to contain the intermediate processing site. The peptidase may therefore cleave the precursor initially at this site and then at the cleavage site that is at the N-terminus of the mature polypeptide. The peptidase has not been purified to homogeneity so we cannot exclude the possibility that two peptidases are required for the processing.

The pea thylakoid processing peptidase was also isolated from thylakoid membranes and, using the wheat 23 kDa and *P. laminosum* 9 kDa precursor polypeptides, the specificities of the pea and *P.*

laminosum peptidases have been compared. As shown previously, the wheat precursor 23 kDa polypeptide is a good substrate for the pea peptidase [7]. We show here that it is also efficiently processed by the *P. laminosum* peptidase. The cyanobacterial peptidase is, therefore, capable of correctly processing a higher plant precursor that has a different primary sequence at the cleavage site. This lends further support to the view that processing is not entirely dependent upon primary sequences and is consistent with previous data that indicates that there is a degree of flexibility in the sequences that a particular peptidase will recognize and cleave [7]. However, despite being a good substrate for the *P. laminosum* peptidase, the precursor 9 kDa polypeptide is processed by the pea peptidase at a site that is clearly different from the intermediate and mature cleavage sites recognized by the *P. laminosum* peptidase. These results indicate that, under these reaction conditions, the specificity of the pea peptidase is not sufficiently flexible to enable it to process all cleavage sites that have the characteristic $(-3, -1)$ motif and that are recognized by the *P. laminosum* peptidase. This suggests that further, more specific, information (e.g. secondary structure) may be required at a cleavage site for it to be recognized and cleaved by a particular peptidase. We conclude that the reaction specificities of the pea and *P. laminosum* thylakoid processing peptidases are similar but not identical.

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REFERENCES

[1] Smeekens, S., de Groot, M., van Binsbergen, J. and Weisbeek, P. (1985) Nature 317, 456–458.
[2] Rother, C., Jansen, T., Tyagi, A., Tittgen, J. and Herrmann, R.G. (1986) Curr. Genet. 11, 171–176.
[3] Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. and Weisbeek, P. (1986) Cell 46, 365–375.
[4] Hageman, J., Robinson, C., Smeekens, S. and Weisbeek, P. (1986) Nature 324, 567–569.
[5] Magner, J.A. (1982) J. Theor. Biol. 99, 831–833.
[6] Wallace, T.P., Stewart, A.C. Pappin, D. and Howe, C.J. (1989) Mol. Gen. Genet. 216, 334–339.
[7] Halpin, C., Elderfield, P.D., James, H.E., Zimmermann, R., Dunbar, B. and Robinson, C. (1989) EMBO J. 8, 3917–3921.
[8] Perlman, D. and Halvorson, H.O. (1983) J. Mol. Biol. 167, 391–409.
[9] Heijne, G. von (1983) Eur. J. Biochem. 133, 17–21.
[10] Kuwabara, T., Reddy, K.J. and Sherman, L.A. (1987) Proc. Natl. Acad. Sci. USA 84, 8230–8234.
[11] Philbrick, J.B. and Zilinskas, B.A. (1988) Mol. Gen. Genet. 212, 418–425.
[12] Homann, P.H. (1987) J. Bioenerg. Biomembr. 19, 105–123.
[13] Stewart, A.C., Ljungberg, U., Åkerlund, H.-E., Andersson, B. (1985) Biochim. Biophys. Acta 808, 353–362.
[14] Stewart, A.C., Siczkowski, M. and Ljungberg, U. (1985) FEBS Lett. 193, 175–179.
[15] Rolfe, S.A. and Bendall, D.S. (1989) Biochim. Biophys. Acta 973, 220–226.
[16] Stewart, A.C. and Bendall, D.S. (1979) FEBS Lett. 107, 308–312.
[17] Kirwin, P.M., Elderfield, P.D. and Robinson, C. (1987) J. Biol. Chem. 262, 16386–16390.
[18] Robinson, C. and Ellis, R.J. (1984) Eur. J. Biochem. 142, 343–346.
[19] Mishkind, M.L., Wessler, S.R. and Schmidt, G.W. (1985) J. Cell. Biol. 100, 226–234.
[20] Heijne, G. von (1986) Nucleic Acids Res. 14, 4683–4690.