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Chloroplasts of the green alga *Chlamydomonas reinhardtii* possess at least four distinct stromal processing proteases

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Abstract

The majority of the proteins in the chloroplast are encoded in the nucleus and synthesised in the cytoplasm as precursors with N-terminal extensions. These targeting sequences guide the precursor proteins into the chloroplast where they are immediately cleaved off by a stromal processing protease (SPP). It is commonly assumed that in higher plant chloroplasts one general SPP processes almost all imported precursor proteins. In the green alga *Chlamydomonas*, however, there exist several different SPPs which process the various *Chlamydomonas* precursor proteins. The seven precursor proteins investigated here, which were all correctly imported into isolated chloroplasts, could be divided into two groups: Four precursor proteins were cleaved correctly when processed *in vitro* with an extract of stromal proteins. Four different SPPs were found in *Chlamydomonas* chloroplasts to be responsible for the processing of this class of precursors and these four activities were separated chromatographically, characterised and further distinguished by their sensitivity to different inhibitors. The three precursors of the second group were degraded completely by unidentified enzyme(s) present in the stromal extract. Degradation of these precursors was dependent on their conformational integrity as well as on the redox state in the stroma.

Abbreviations: dpm – disintegrations per minute, DTT – dithiothreitol, *iSS – *in vitro* expressed intermediate form of precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase lacking the 24 N-terminal amino acids, pcytc – precursor of cytochrome c552, pOEE1 – precursor of subunit 1 of the oxygen evolving enzyme, pPC – precursor of plastocyanin, pPS I-F, H, K – precursors of subunit F, H, K of Photosystem I, SPP – stromal processing peptidase, pSS – precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase, TLCK – tosyl-lysylchloromethane, TPCK – tosyl-phenylalanylchloromethane, TPP – thylakoid processing peptidase

Introduction

Most chloroplast proteins are encoded in the nucleus and synthesised on cytoplasmic ribosomes as precursor proteins with an N-terminal extension or transit peptide. This targeting sequence contains all the information needed to guide the protein into the chloroplast (De Boer and Weisbeek 1991; Wan et al. 1996; Soll and Tien 1998). As soon as the transit peptide reaches the stroma, it is specifically cleaved off by the soluble stromal processing protease (SPP); hence the processing is probably co-translocational (Schnell and Blobel 1993). In import assays using isolated higher plant chloroplasts, competition experiments with different precursor proteins revealed that there is only one general import pathway for nearly all precursor proteins (Buvinger et al. 1989; Perry et al. 1991). Thus, it was assumed that there is only one processing protease in the stroma which was intriguing as diverse precursor proteins display little or no primary se-

quence similarity (Levy and Adam 1995; Lübeck et al. 1997). Several groups, using partially purified protease activities (Robinson and Ellis 1984; Abad et al. 1991; 1995) confirmed this assumption in higher plants. A general chloroplast processing protease (CPE) of 140 kDa was highly purified and found to be a metalloprotease (Oblong and Lamppa 1992). CPE cleaved a variety of precursors of functionally different proteins and from different species correctly. The presumptive gene was cloned (VanderVere et al. 1995) and the protein expressed in *E. coli* was able to process different precursor proteins from various origins (Richter and Lamppa 1998). The introduction of the antisense-CPE gene into the cell genome resulted in severe pleiotropic effects on chloroplast structure and development (Wan et al. 1998). However, two recent reports suggest that this concept of a general protein import machinery in higher plants should be modified slightly. Evidence was found for developmental variants of the general import apparatus (Jarvis et al. 1998) and for the existence of SPPs other than the general processing protease (Kossuevitzky et al. 1998).

Protein import into lower plant chloroplasts has never been studied, while only a few reports have dealt with *in vitro* protein import into isolated chloroplasts from algae. Studies with the green alga *Chlamydomonas reinhardtii* (Franzén et al. 1989; Hugosson et al. 1995) showed there were interesting differences between the protein import systems of algae and higher plants (Rothen et al. 1997). *Chlamydomonas* transit peptides show closer homologies to presequences of mitochondrial precursor proteins than to chloroplast precursors of higher plants (Franzén et al. 1990). In addition, secondary structure predictions indicate differences between *Chlamydomonas* and higher plant transit peptides (Franzén 1994). In agreement with this, we showed that the processing of the transit peptide obeys different rules in *Chlamydomonas vs.* higher plant chloroplasts (Su and Boschetti 1994). The transit peptides of precursor proteins from higher plants were not cleaved correctly by a stromal extract of *Chlamydomonas* chloroplasts and *vice versa*. Fractionation of stromal proteins from *Chlamydomonas* revealed for the first time the existence of more than one processing enzyme in chloroplasts. The precursor of the small subunit of the stromal protein ribulose-1,5-bisphosphate carboxylase (pSS) was found to be a substrate for two proteolytic enzymes, SPP-1 and SPP-2. SPP-2 removed the complete transit peptide of pSS, whereas

SPP-1 generated an intermediate form iSS by cleaving within the transit peptide. A third stromal protease was found which processed the transit sequence of the protein-subunit 1 (pOEE1) of the oxygen-evolving complex of PS II which is located in the thylakoid lumen. pOEE1 was cleaved to an intermediate form and its final maturation was carried out by a thylakoid processing peptidase (TPP) residing within the lumen (Su and Boschetti 1993).

In recent times more cDNA clones encoding *Chlamydomonas* precursor proteins have become available. In the present study seven precursor proteins were synthesised *in vitro* and employed as substrates to detect new stromal processing proteases with different substrate specificities in *Chlamydomonas* chloroplasts. The seven precursor proteins studied could be divided into two groups: Four were cleaved to produce distinct products in an *in vitro* assay involving incubation with an extract of stromal proteins and four specific proteases were found to be responsible for this *in vitro* processing. The precursors of the second group were unstable and completely degraded in the same assay. These unstable precursor proteins seemed to be degraded by a sulphydryl-dependent protease or oxidase present in the stromal extracts. The conformation of these three proteins might also affect their stability.

Materials and methods

All experiments were performed with a cell-wall deficient mutant *cw*-15 of *Chlamydomonas reinhardtii*, which was obtained as strain CC-277 from Dr E. Harris (Chlamydomonas Genetics Centre, Duke University, Durham, USA). Chloroplasts were isolated according to the method of (Mendiola-Morgenthaler et al. 1985).

Synthesis of precursors

Plasmids containing the genes for the precursors of the following proteins were obtained from different laboratories: SS (stromal protein) (M. Mishkind); OEE1 (lumenal protein) (J. D. Rochaix); cytochrome-c552 and plastocyanin (lumenal proteins) (cytc, PC; S. Merchant); subunits F, H and K of Photosystem I (thylakoid membrane proteins) (PS I-F, H, K; L. G. Franzén). The plasmid encoding *iSS was constructed by excising from the original plasmid (Anderson and Smith 1986) the coding sequence encoding the 24

N-terminal amino acids of the transit peptide with PstI and NcoI. Before religation, the ends were made blunt with Klenow. The cDNA clone was linearised with PvuII before transcription and translation was performed as for pSS. cDNA clones encoding the *Chlamydomonas* precursors PS I-F, PS I-H and PS I-K, as well as cytc and PC were linearised before being transcribed by T7 polymerase (Boehringer Mannheim AG, Germany). mRNA was translated in a wheat germ system prepared according to Mishkind et al. (1987), in the presence of $[^{35}S]$-methionine (Amersham International plc). Alternatively, linearised clones were used for protein synthesis in a coupled transcription-translation rabbit reticulocyte lysate system (Promega Biotech., USA) in the presence of $[^{35}S]$-methionine.

The procedures for synthesis of pSS and pOEE1 and import of radiolabelled precursor proteins into intact chloroplasts were described in Su et al. (1992).

Preparation of stromal extracts

Stromal protein extracts were prepared according to Su and Boschetti (1994). Briefly, intact isolated chloroplasts were lysed in 50 mM HEPES pH 8.0 at a chloroplast concentration of $1-2 \times 10^{9}$/ml at 4 °C for 30 min and subsequently centrifuged at $16000 \times g$ for 30 min at 4 °C. Stromal supernatant was further centrifuged at $100000 \times g$ for 1 h to remove ribosomes. The processing activity remained in the soluble fraction.

Fractionation of stromal extracts

Fractionation of stromal proteins on Superose-G6 and Mono-Q columns was performed as previously described (Su and Boschetti 1993). Briefly, stromal extract was loaded on a FPLC-Superose-G6 column (Pharmacia). Fractions of 200 μl were collected and every second fraction tested for processing activity. Where necessary, four successive fractions were pooled and concentrated 16 times in Centricon 10 concentrators (Amicon Inc.) before being tested for processing activity.

From four individual runs on Superose-G6 the fractions with maximal processing activities were pooled and applied to an FPLC-Mono-Q HR column pre-equilibrated with 25 mM Tris-HCl pH 9.0. The column was eluted with a linear salt gradient (from 0 to 1 M KCl) in 25 mM Tris-HCl pH 9.0. The fractions with the highest enzyme activities were divided into small aliquots and stored at -80 °C. No loss of processing activity was observed within two months.

In vitro *processing assay*

The processing activities of individual chromatographic fractions were assayed by incubation with *in vitro* synthesised precursor proteins. Incubation mixtures consisted of one volume of translation mixture containing radiolabelled precursor protein equivalent to 100000 dpm, (if necessary, the volume was adjusted with 50 mM Tris-HCl pH 9.0) and one volume of chloroplast stromal extract. After incubation for 90 min at 25 °C, the reaction was stopped by the addition of one volume of sample buffer and then heated for 3 min at 90 °C. Samples were analysed by SDS-PAGE and fluorography.

Inhibition of protease activity was determined as described in Su and Boschetti (1993). Briefly, mixtures of 10 μl 50 mM Tris-HCl pH 9.0 containing protease inhibitors and 10^{5} dpm of the $[^{35}S]$-labelled precursor proteins were incubated with 10 μl of the respective partially purified stromal protease. The final concentration of all inhibitors was 5 mM, with the exception of TPCK and TLCK (0.5 mM) and *p*-aminobenzamidine (1 mM). After incubation at 25 °C for 90 min samples were analysed as described.

In some assays containing the precursors pPS I-F, H and pcytc, DTT was added to the incubation mixtures at a final concentration of 0.1 M. Where indicated, after the translation reaction the precursors were unfolded by addition of urea to the wheat germ extract to a concentration of 6 M, then diluted into the incubation assays to produce a final concentration of 0.5 M urea. All assays containing DTT or urea were checked for correct pH value.

Results

Selection of precursor proteins used as substrates for in vitro *processing in stromal extracts*

Precursors of *Chlamydomonas* chloroplast proteins were first screened for their ability to be efficiently transcribed *in vitro* and translated in a wheat germ system and to be labelled to a high specificity with $[^{35}S]$-methionine. The precursor proteins were then tested for successful *in vitro* import into isolated *Chlamydomonas* chloroplasts and for specific processing within these intact chloroplasts. By these

criteria, in addition to the previously studied pSS and pOEE1 (Su and Boschetti 1994), three precursor proteins of subunits of the Photosystem I and the precursors of plastocyanin and cytochrome c552 were chosen as substrates for further investigation.

In a preliminary experiment, we tested whether the *in vitro* synthesised precursor proteins were cleaved *in vitro* by a crude stromal extract. Stromal extracts were prepared as described in 'Materials and methods' and incubated with the radioactively labelled precursor proteins. The incubation mixtures were then analysed by SDS-PAGE and fluorography for specific cleavage products. The molecular weights of the cleavage products were always compared to the molecular weights of the mature of intermediate proteins generated upon import into intact chloroplasts. As expected, pPS I-K and pSS were cleaved specifically to their mature form, while pOEE1 and pPC were cleaved to their intermediate form (Figure 1). Interestingly, a subset of *Chlamydomonas* precursor proteins, including pcytc, pPS I-F and pPS I-H, proved to be unstable when incubated with an extract of stromal proteins and no specific cleavage products were generated from these precursor proteins.

Specific processing proteases separated by size-exclusion chromatography

Previously, using the two precursor proteins pSS and pOEE1 as substrates, fractionation of stromal proteins according to their size revealed the presence of three strictly substrate specific processing activities (Su and Boschetti 1993; 1994). The availability of more labelled precursor proteins now allowed us to test for the existence of additional stromal processing proteases which might specifically cleave these precursors. Chloroplast stromal proteins were separated on a Superose-G6 column and each second fraction was analysed for its ability to process the new substrates, namely the precursors of plastocyanin (pPC) and subunit K of Photosystem I (pPS I-K). To allow comparison with previous experiments, all fractions were also tested with pSS. Figure 2B shows that fractions 14 to 24 contained predominantly SPP-2 which cleaves pSS to its mature form. The second proteolytic activity SPP-1, which specifically cleaves pSS to an intermediate form iSS, mainly eluted in fractions 8–12. The intermediate formed in the late fractions 24–30 is not identical with iSS. The physiological relevance of the formation of iSS remains unknown to date.
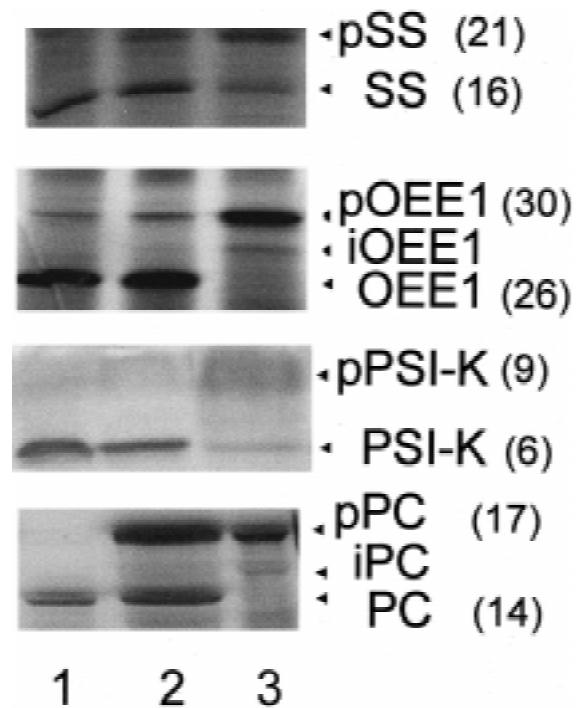

Figure 1. Comparison of processing of the precursors pSS, pOEE1, pPS I-K and pPC in intact chloroplasts and *in vitro* by stromal extracts of *Chlamydomonas* chloroplasts. Lane 1: *in vitro* synthesised and radiolabelled precursor proteins were imported into intact chloroplasts. After reisolation by pelleting through a Percoll cushion the chloroplasts were treated with thermolysin solubilised and the proteins analysed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane 2: Same as in lane 1 but without thermolysin treatment. Lane 3: Precursor proteins incubated with an extract of stromal proteins. Each picture represent a scanned image of the fluorogram of one SDS-polyacrylamide gel, however, not relevant parts of the gel were removed by image processing. Precursors, intermediate and mature forms are indicated by arrow heads. In parenthesis: relative molecular mass (kDa).

Figure 2C demonstrates that pPC, which has a bipartite transit sequence, was processed to its stromal intermediate form iPC when incubated with fractions number 14 to 24 from the Superose-G6 column. The diffuse band slightly below pPC, which is present at constant intensity in all fractions as well as in the starting material, is considered to be a side product of translation in the wheat germ extract. Obviously, pPC was cleaved by a protease which eluted in exactly the same fractions as SPP-2 activity. The intermediate form iPC will serve as a substrate for a crude TPP preparation as previously demonstrated (Rüfenacht and Boschetti 1997).

By using the precursor of the intrinsic membrane protein pPS I-K as a substrate a further processing
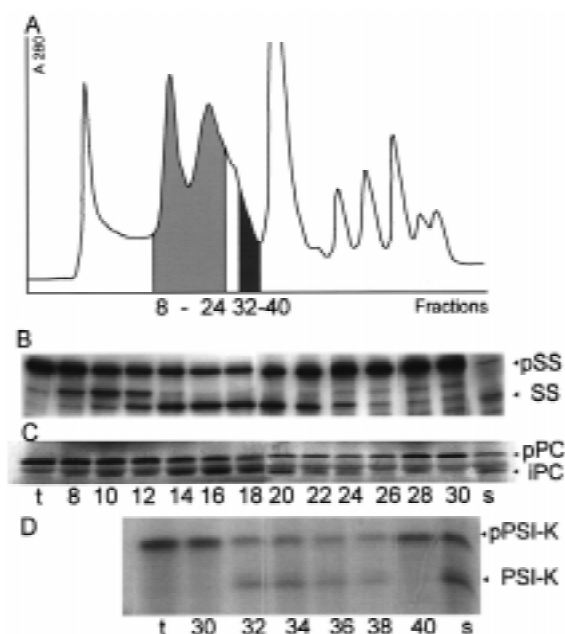
Figure 2. Separation by size-exclusion chromatography of activities which process pSS, pPC and pPS I-K. Stromal proteins from isolated intact *Chlamydomonas* chloroplasts were separated on a Superose-G6 column. The absorbency profile recorded at 280 nm and the fractions that were taken are depicted in A. Every second fraction was assayed for processing activity with pSS (B) and pPC (C). Fractions were concentrated before incubation with pPS I-K (D). After incubation, samples were subjected to SDS polyacrylamide gel electrophoresis and fluorography. The first lane on the left shows translation product only (t), while lanes on the right show processing with whole stromal extract (s). The respective fractions that were tested are indicated by their numbers. Arrowheads show the position of precursor (pSS, pPC, pPS I-K), intermediate (iPC) and mature proteins (SS, PS I-K), respectively.

protease was found in *Chlamydomonas* chloroplasts. Figure 2D shows that the specific processing activity SPP-PS I-K eluted in fractions 32 to 40 and was completely separated from fractions containing SPP-2. SPP-PS I-K specifically processed the transit peptide of the precursor of subunit K, producing the mature form PS I-K. For all tested precursor proteins processing activities were found exclusively in those fractions represented in Figure 2. No specific processing products could be detected in assays with the other chromatographic fractions.

Separation by anion-exchange chromatography of the activities which process pSS and pOEE1

Previous experiments suggested that pSS and pOEE1 were not processed by the same enzyme. However,

simple size-exclusion chromatography resulted in only a partial separation of the two processing activities SPP-2 and SPP-OEE1 (Su and Boschetti 1994). Therefore, fractions from size-exclusion chromatography enriched in processing activity for both precursor proteins were pooled and further subjected to anion-exchange chromatography on a Mono-Q column. Figure 3 shows that by this method the two processing activities were completely separated. Fractions 1–3 contained a processing activity which specifically cleaved pOEE1 to its intermediate form iOEE1 (Figure 3C, lanes 1–3). The intermediate iOEE1 could be further processed by a crude preparation of TPP to produce mature OEE1 (Rüfenacht and Boschetti 1997). Stromal protein fractions containing SPP-OEE1 activity did not cleave pSS. The protease which processes pSS to its mature form, was detected in fractions eluting at higher salt concentrations (Figure 3B, lane 5). These fractions did not process pOEE1.
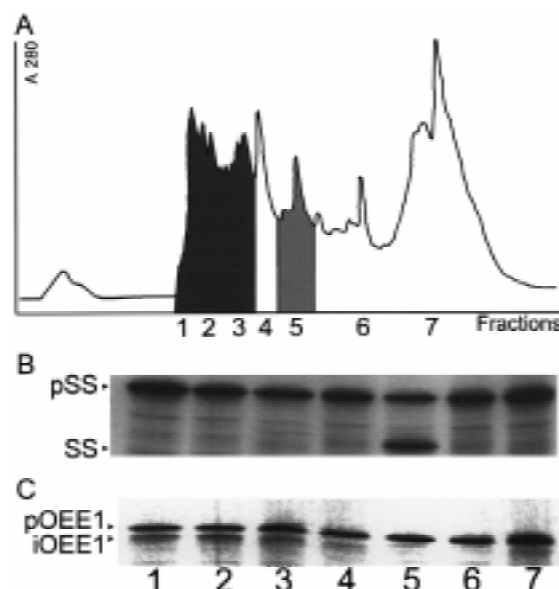

Figure 3. Separation by anion-exchange chromatography of the activities which process pSS and pOEE1. From the stroma of isolated *Chlamydomonas* chloroplasts processing activities were purified first by chromatography on Superose-G6. Fractions 14-24 (Figure 2) were further separated by anion-exchange chromatography on a Mono-Q column. Fractions from the anion-exchange separation were tested either with pSS (B) or pOEE1 (C) as a substrate. After incubation, samples were subjected to SDS polyacrylamide gel electrophoresis and fluorography. Arrowheads indicate the position of precursor (pSS, pOEE1) or processed proteins (SS, iOEE1), respectively. (A) shows the elution profile at 280 nm (A 280). Fractions tested are indicated by numbers.

*Processing of *iSS with N-terminally truncated transit peptide*

To gain more information about the processing activity of SPP-1, we tested whether the N-terminal part of a transit peptide was necessary for *in vitro* processing. We constructed a clone encoding the truncated protein *iSS which lacked the first 24 amino acids of the transit peptide of SS (Figure 4, lane 1 compared to lane 6). Figure 4, lanes 2 and 4 show that as expected pSS, but not *iSS was processed by SPP-1 *in vitro*, since in *iSS the cleavage site was deleted. The truncated precursor protein *iSS, however, was a good substrate for SPP-2 which processed it to yield mature SS (Figure 4, lane 3). SPP-2 cleaved *iSS and pSS with similar specific activities (Figure 4, lane 5). In conclusion, these data show that the N-terminal part of the transit peptide of pSS is not necessary for correct *in vitro* processing by SPP-2.

Characterisation of the different processing activities

To distinguish the different stromal processing activities found in *Chlamydomonas* chloroplasts, their temperature and pH values for maximal processing activity were determined. Figure 5, panel A and B show the temperature and pH range, respectively of two SPPs, namely the enzymes which process pSS
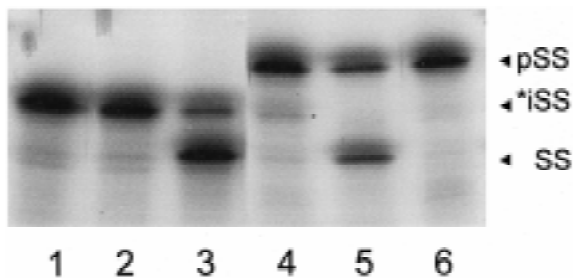
(dots) and pPS I-K (circles). The data were obtained using size-exclusion chromatographic fractions which contained maximal SPP-2 and SPP-PS I-K activities, respectively, and are mean values of three independent experiments. Figure 5 shows that the ranges and the maxima for the two processing reactions are similar, which reflects the fact that these enzymes carry out a similar reaction in the same compartment. Both enzymes show activity over a broad range of temperature and pH values, so that subtle differences that might exist between them would be difficult to detect.
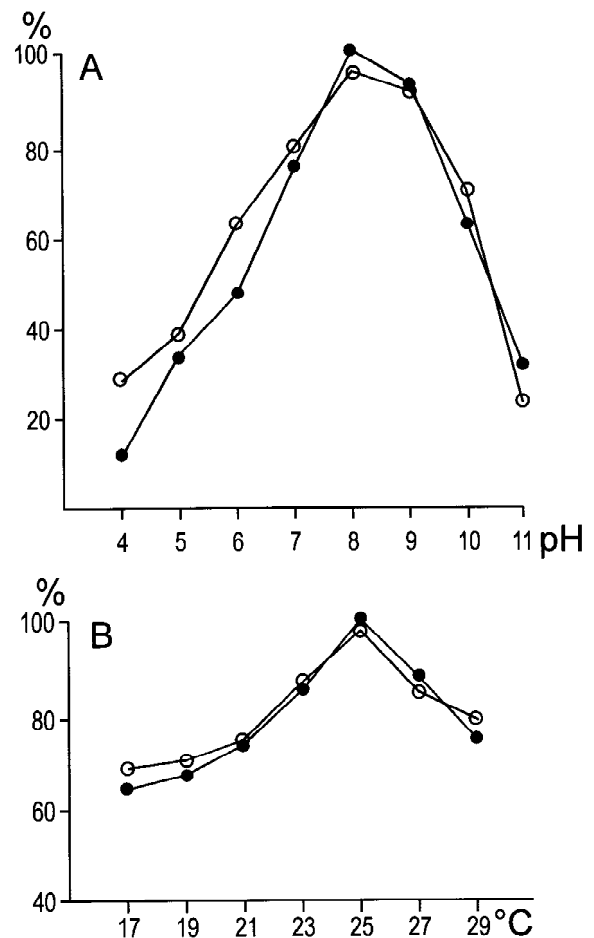

Figure 4. Specific processing of *iSS, a truncated form of pSS lacking 24 N-terminal amino acids. Partially purified SPP-2 and SPP-1 preparations were incubated with the radiolabelled precursors. For SPP-2 fractions 17-23 of Figure 2 were pooled, while SPP-1 consisted in fractions 7 and 8 of Figure 2 containing low SPP-1 activity, but being hardly contaminated with SPP-2. The starting materials were *iSS (lane 1) and pSS (lane 6), respectively. Lane 2: *iSS incubated with SPP-1 and lane 4: pSS incubated with SPP-1. Lane 3: *iSS incubated with SPP-2 and lane 5: pSS incubated with SPP-2. This figure represents a scanned image of the fluorography of one SDS-polyacrylamide gel, however, the central part of the gel was removed by image processing.

Figure 5. pH and temperature optima of the specific processing activities SPP-2 and SPP-PS I-K. Partially purified SPP-2 (dots) and SPP-PS I-K (circles) preparations pooled after anion-exchange and size-exclusion chromatography, respectively, were incubated with the radiolabelled precursors pSS and pPS I-K. After incubation, samples were subjected to SDS-polyacrylamide gel electrophoresis and exposed to PhosphorImager plates. Quantitative analysis of the samples was performed using the ImageQuant software.

Effect of inhibitors on the stromal processing proteases

To further establish the specificity and nature of the stromal processing proteases we tested their sensitivity to various protease inhibitors. Such assays have already been performed with SPP-1, SPP-2 and SPP-OEE1 using crude stromal preparations (Su and Boschetti 1993). Figure 6 shows the effect of various inhibitors on the processing of pSS, pPC and pPS I-K. pSS and pPC were incubated in the presence of the indicated inhibitors with pooled fractions of stromal proteins (containing maximal processing activity) recovered after anion-exchange chromatography. pPS I-K was tested with concentrated stromal protein fractions, recovered and pooled after size-exclusion chromatography.

Figure 6B shows that the previous findings about inhibition of pSS (Su and Boschetti 1993) could now be reproduced with further purified SPP-2 preparations. SPP-2 activity was mainly inhibited by TPCK, which specifically blocks serine- and cysteine-proteases (Figure 6B, lane 8). EDTA had no effect on SPP-2 (Figure 6B, lane 4), which shows that this enzyme is not a metalloprotease. Interestingly 1,10-phenanthroline was able to inhibit SPP-2 activity completely (Figure 6B, lane 6). 1,10-phenanthroline
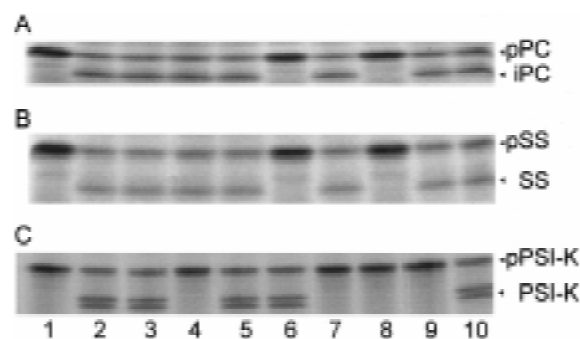

Figure 6. Effects of protease inhibitors on SPP-2 (A, B) and SPP-PS I-K (C) activities. Radiolabelled pPC (lane 1) was incubated with the peptidase SPP-2 in the absence of inhibitors (lane 2), and at a concentration of 2.5% ethanol (lane 3), 5 mM EDTA (lane 4), 5 mM EGTA (lane 5), 5 mM 1,10-phenanthroline (lane 6), 0.5 mM TLCK (lane 7), 0.5 mM TPCK (lane 8), 1 mM p-aminobenzamidine (lane 9), and 5 mM DTT (lane 10). (B): same as in (A) but with pSS as the substrate. (C): same as in (A, B), but with SPP-PS I-K and pPS I-K as the substrate. After incubation, samples were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Arrowheads show the position of precursor (pSS, pPC, pPS I-K), intermediate (iPC) and mature proteins (SS, PS I-K), respectively.

is known to inhibit metalloproteases (Whelan et al. 1996), but there are also reports that it can inhibit enzymes in a non-specific manner (Wu et al. 1995).

Figure 6A shows the processing of the precursor protein pPC. Again, 1,10-phenanthroline and TPCK were the only inhibitors of the cleavage of the transit peptide of pPC. Thus, pPC processing was performed by an enzyme sensitive to the same inhibitors as SPP-2. Taken together, these data suggest that pSS and pPC are processed by the same enzyme and that this enzyme is a serine or cysteine type protease.

The processing of the precursor protein pPS I-K was inhibited not only by TPCK and TLCK but also by *p*-aminobenzamidine (Figure 6C). Processing was also completely abolished in the presence of EDTA (Figure 6C, lane 4), which indicated that this enzyme has an extra requirement for metal-ions, but not for calcium as EGTA shows no effect. Why in the experiment of Figure 6C, but not of Figures 1 and 2, mature PS I-K migrated as a double band is not clear. It might reflect an artefact of the electrophoretic separation.

In vitro *processing of the precursor proteins pPS I-F, pPS I-H and pcytc*

When incubated with a crude stromal extract, these three precursor proteins were digested without generation of distinct cleavage products (Figure 7). We assumed that the complete digestion of these precursors was due to a non-specific protease present in stromal extracts of *Chlamydomonas* chloroplasts. To minimise non-specific degradations, we tried to optimise incubation conditions, e.g. to shorten the incubation time. Only incubations in the presence of dithiothreitol, (lane 4) led to a stabilisation of the three precursor proteins, although still no specific processing products were detected. Obviously, the enzyme responsible for the non-specific degradation of the precursor proteins was blocked by the addition of DTT, while in control assays the SPP-2 activity was not affected (A. Rüfenacht and A. Boschetti, unpublished data).

Processing of precursor proteins may sometimes be stimulated by the addition of urea to the incubation. We thus denatured the precursors of subunits F and H and cytc with 6 M urea. Addition of the denatured precursors (in 0.5 M urea, 0.1 M DTT) to the stromal extracts did not lead to the appearance of distinct cleavage products but abolished the protective action of DTT (Figure 7, lane 6). The reason for this susceptibility is not clear.
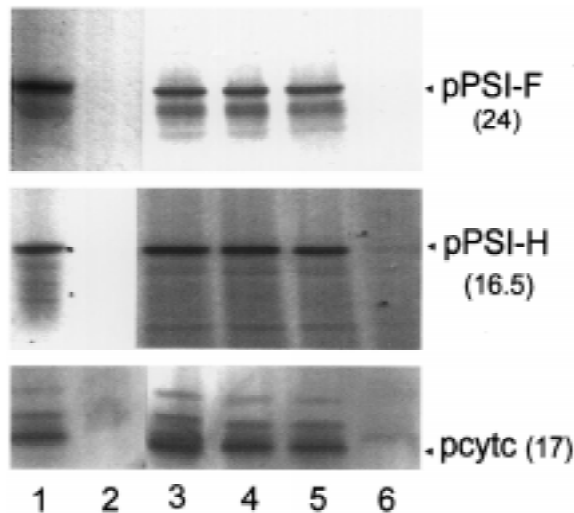
Figure 7. Stability of the precursors pPS I-F, pPS I-H and pcytc in stromal extracts of *Chlamydomonas* chloroplasts. In lanes 1, 3 and 5, for comparison the radiolabelled precursors pPS I-F, pPS I-H and pcytc were analysed by SDS-polyacrylamide gel electrophoresis and fluorography (100000 dpm per lane). In lane 2 pPS I-F, pPS I-H and pcytc (100000 dpm per assay) were incubated with a crude stromal extract prepared from intact isolated chloroplasts of *Chlamydomonas* and the whole assays were subjected to electrophoresis. Lane 4 shows effects of DTT when present in the incubations at a final concentration of 0.1 M. In lane 6 the radiolabelled precursors were unfolded in 6 M urea, then added to the incubation mixtures, which contained 0.1 M DTT, to produce a final concentration of 0.5 M urea. Each picture represents a scanned image of the fluorogram of one SDS-polyacrylamide gel, however, not relevant parts of the gel were removed by image processing. In parenthesis: relative molecular mass (kDa).

In conclusion, of the seven precursor proteins tested the four listed in Table 1 were specifically processed *in vitro* to yield their mature or stromal intermediate form by the action of three different SPPs. A fourth SPP cleaved pSS specifically at a site within the transit sequence. All these processing enzymes were completely separated by chromatography and showed different patterns of sensitivity to various protease inhibitors (Table 1).

Table 1. Products formed in vitro from precursor proteins by the specific stromal processing proteases found in chloroplasts of *Chlamydomonas reinhardtii*

	SPP-1	SPP-2	SPP-OEE1	SPP-PS I-K
pSS	iSS	SS	—	—
pPC	—	iPC	—	—
pOEE1	—	—	iOEE1	—
pPS I-K	—	—	—	PS I-K

Discussion

In higher plants a general stromal processing protease was found which has a very broad substrate specificity and is assumed to be responsible for the correct cleavage of most chloroplast precursor proteins (Richter and Lamppa 1998).

In contrast, in *Chlamydomonas* we have previously shown the existence of three proteases, two of which, namely SPP-2 and SPP-1, cleaved pSS *in vitro* at specific sites and showed different sensitivities to various protease inhibitors (Su and Boschetti 1993) while the third protease processed pOEE1 (Su and Boschetti 1994). We now report the complete chromatographic separation of the two specific activities which process pSS and pOEE1.

The availability of cDNA encoding additional *Chlamydomonas* precursor proteins led us to the discovery of a further processing activity in the stroma of *Chlamydomonas* chloroplasts. By a single chromatographic step on a size-exclusion column, protein fractions were obtained which cleaved exclusively pPS I-K to a distinct form of lower molecular weight, but processed none of the other experimentally available precursor proteins. As judged by high resolution gel electrophoresis, this product was of the same size as mature PS I-K formed during an *in vitro* import assay into intact chloroplasts, supporting our assumption that the processing activity was indeed due to an enzyme which was specific for the transit peptide of pPS I-K. This enzyme is further distinguishable from the other processing activities by its different pattern of protease inhibitors.

On the other hand there is strong evidence that pPC is cleaved by the same processing enzyme SPP-2 which cleaves also pSS to the mature form SS. The protease SPP-2 might be a processing enzyme which displays less strict substrate specificity than the other SPPs. In support of this hypothesis only the C-terminal part of the transit peptide of pSS is required to allow processing *in vitro*. The observation that *iSS, which is almost of the same molecular size as the intermediate form of pSS generated by the SPP-1 activity, is cleaved with the same specific activity as pSS might indicate that processing of the precursor protein pSS occurs *via* a two step mechanism, although we cannot rule out that cleavage of pSS by SPP-1 within the transit peptide is simply a by-path of processing.

In contrast to higher plants, which apparently contain one general processing protease, our studies

revealed the existence of already four specific proteases using only four precursor proteins in *Chlamydomonas* as test substrates. This difference between green algae and higher plants is in good agreement with previously reported differences in chloroplast protein import (Hugosson et al. 1995; Rothen et al. 1997). *Chlamydomonas* SPPs display a higher degree of substrate specificity than the higher plant SPP and most likely belong to the family of serine-proteases.

Three other *in vitro* synthesised precursor proteins of *Chlamydomonas* (pcytc, pPS I-F and pPS I-H) do not yield distinct cleavage products, but rather are degraded rapidly when treated with stromal extracts *in vitro*. When imported into intact chloroplasts this class of precursors of thylakoid proteins is correctly processed to stable mature forms. Other groups working with higher plants demonstrated processing of such precursor proteins as e.g. pPS I-F from barley not only in intact chloroplasts (Mant et al. 1994; Karnauchov et al. 1994), but also *in vitro* (Scott et al. 1994). We assume that in *Chlamydomonas* pcytc, pPS I-H and pPS I-F eventually belong to a group of precursor proteins which are not processed in the stroma, but are directly imported into thylakoids and processed by lumenal peptidases (Nielsen et al. 1994; Michl et al. 1994; Lorkovic et al. 1995). It might also be that the very fast action of a non-specific protease or other degrading enzyme present in the stromal extracts obscured the detection of a presumptive substrate-specific processing which eventually was slowed down by incorrect folding of these substrates. The results we obtained in the presence of DTT suggest that such a non-specific enzyme is dependent on the redox conditions in the stromal extracts, a phenomenon that has previously been reported for the degradation of other chloroplast proteins (Zer et al. 1994). Rapid degradation, involving ATP dependent ClpP proteases, of misrouted nuclear encoded thylakoid proteins has also been reported (Halperin and Adam 1996). It is possible that these three precursors of thylakoid membrane-derived proteins are unstable in a membrane-free environment. The analogous protein psaD of PS I was reported to become resistant to proteases only after the precursor is processed to its mature form following insertion into the PS I complex (Minai et al. 1996). More work will be needed to elucidate the specific processing characteristics of *Chlamydomonas* pPS I-F, pPS I-H and pcytc proteins.

The fact that in *Chlamydomonas* chloroplasts several SSPs with different specificities were found, while in higher plants the so called general processing protease exhibits a very broad substrate specificity (Richter and Lamppa 1998), might reflect the interesting differences reported between algal and green plant chloroplast protein import (Robinson and Ellis 1984). Eventually some of the diverse new proteases with as yet unknown functions, found within chloroplasts in the last few years reviewed in Adam (1996), might also be shown to act as specific processing enzymes in higher plants.

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