Chloroplast Biology

IDENTIFICATION OF NUCLEUS-ENCODED FACTORS INVOLVED IN THE ACCUMULATION OF SPECIFIC CHLOROPLAST RNAS OF CHLAMYDOMONAS

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Genetic analysis has revealed that the accumulation of several chloroplast mRNAs of the green alga *Chlamydomonas reinhardtii* requires specific nucleus-encoded functions. We have characteized three nuclear mutants, nac2, 222E and F24 which lack *psbD*, *psbB* and *psaB* mRNA, respectively [1, 2]. In each case we have shown that the target of the nucleus-encoded function affected in these mutants is the 5' untranslated region (5'UTR) of the corresponding mRNA [3, 4, 5]. Site-directed mutagenesis has led to the identification of several cis-acting elements required for RNA stability and/or translation [4, 5]. We have cloned the nuclear *Nac2* and *Mbb1* genes by rescue of the mutants which are deficient in the accumulation of the mRNAs of *psbD* or the *psbB/psbT/psbH* chloroplast transcription unit, respectively. *Nac2* encodes a hydrophilic polypeptide of 1385 amino acids with 9 tetratricopeptide repeats (TPR) in its C-terminal half whereas Mbb1 encodes a polypeptide of 662 amino acids which also contains 9 TPRs. TPRs have been identified in many proteins of different function and appear to be involved in protein-protein interactions. Both Nac2 and Mbb1 contain a putative chloroplast transit peptide at their N-terminal end. These proteins have been tagged with the HA epitope and localized in the stromal compartment of the chloroplast. Nac2 and Mbb1 are part of high molecular weight complexes of 500 and 250 kDa, respectively, which is associated with RNA in the case of Nac2. A change of a conserved Ala residue of the fourth TPR motif by site-directed mutagenesis completely abrogates Nac2 function indicating that this TPR is important for *psbD* mRNA stability, processing and/or translation. To test for genetic interactions between the three nuclear loci affected in the nac2, 222E and F24 mutants we have constructed double mutants. Accumulation profiles of *psbD*, *psbB* and *psaB* mRNA were additive in all cases and no synergistic effects could be detected suggesting that the nucleus-encoded factors act to a large extent independently from each other.

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CHLOROPLAST RNA *TRANS*-SPLICING: THE ROLE OF NUCLEUS-ENCODED FACTORS.

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Chloroplast biogenesis is governed by two genomes, one in the organelle and the other in the nucleus. Chloroplast development, and in particular the assembly of the photosynthetic complexes in the thylakoid membrane, requires the coordinate expression of genes in the two compartments. The unicellular green alga *Chlamydomonas reinhardtii* is a facultative phototroph, so that many mutants with defects in photosynthetic function can be recovered. The mutations map either to the chloroplast genome or to nuclear loci. Some of the mutations cause defects in the structural genes for components of the chloroplast such as subunits of the photosystems or enzymes involved in carbon fixation. Other mutations affect chloroplast gene expression and reveal *trans*-acting factors that are required in post-transcriptional steps such as RNA splicing, mRNA stability or translation. The mutations are surprisingly specific since they usually affect the expression of a single chloroplast gene or gene cluster. They reveal a large number of nuclear loci required for the expression of the chloroplast genome. We have investigated factors that are required for splicing of the *psaA* mRNA, which encodes one of the major subunits of PSI.

In *C. reinhardtii*, the *psaA* gene is composed of three exons widely scattered in the circular chloroplast genome. They are transcribed separately as precursors which are then assembled by a process that requires two steps of splicing *in trans*. The split introns share many of the conserved structural features of group II introns, but an additional small chloroplast RNA (product of *tscA*) is required for *trans*-splicing of the first split intron (2). At least 14 nuclear loci are required specifically for the *trans*-splicing of one, the other or both of the *psaA* split introns (1).

Using insertional mutagenesis or mutant rescue with cosmid libraries, we have identified and cloned three novel nuclear genes required for *psaA trans*-splicing. *Maa1* and *Maa2* are required for *trans*-splicing of the second intron of *psaA*, *Maa3* for *trans*-splicing of the first intron. One of the factors, Maa2, shares a high degree of similarity with conserved domains of pseudouridine synthases (3). However site-directed mutagenesis suggests that this catalytic activity is not required for the function of Maa2 in *psaA trans*-splicing. Maa2 may thus derive from a pseudo-uridine synthase that could have been recruited during evolution to facilitate splicing. The two other factors, Maa1 and Maa3, are novel proteins with unusual structural features.

The amount of Maa2 is reduced in some of the other mutants deficient in *trans*-splicing. Maa1 and Maa2 are associated with a membrane fraction of the chloroplast, can be immuno-precipitated together, and sediment in the same fractions in sucrose gradients. This genetic and biochemical evidence indicates that Maa1 and Maa2 may be part of a large complex involved in *trans*-splicing.

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THE TOC159 FAMILY OF CHLOROPLAST PROTEIN : IMPORT RECEPTORS IN ARABIDOPSIS

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Light triggers the developmental program in plants leading to the biogenesis of photosynthetically active chloroplasts from nonphotosynthetic proplastids. During chloroplast biogenesis the photosynthetic apparatus is rapidly assembled, mostly from nuclear-encoded imported proteins. These are synthesized in the cytosol as precursors with cleavable N-terminal targeting sequences (transit sequences). The targeting of nuclear-encoded proteins to the chloroplast is mediated by protein translocon complexes at the outer (Toc complex) and inner (Tic complex) envelope membranes. The pea Toc complex consists of three major components, Toc159, Toc34 and Toc75. Toc159 is an integral membrane GTP-binding protein that appears to function as the major receptor for preproteins at the chloroplast surface. To investigate the roles of Toc159 in vivo and study the role of import in plastid biogenesis, we sought to identify and manipulate the corresponding Toc159 gene in *Arabidopsis thaliana*. An analysis of the available Arabidopsis genomic sequence databases reveals the existence of a family of three putative import receptor proteins related to pea Toc159 (psToc159). We designate these putative receptor proteins, atToc159, atToc132 and atToc120 on the basis of their deduced molecular masses in kilodaltons. Protein import and coimmunoprecipitation studies confirm that all three proteins are authentic Toc components. We will present evidence that atToc159, -132 and-120 play specific, essential roles in chloroplast biogenesis.

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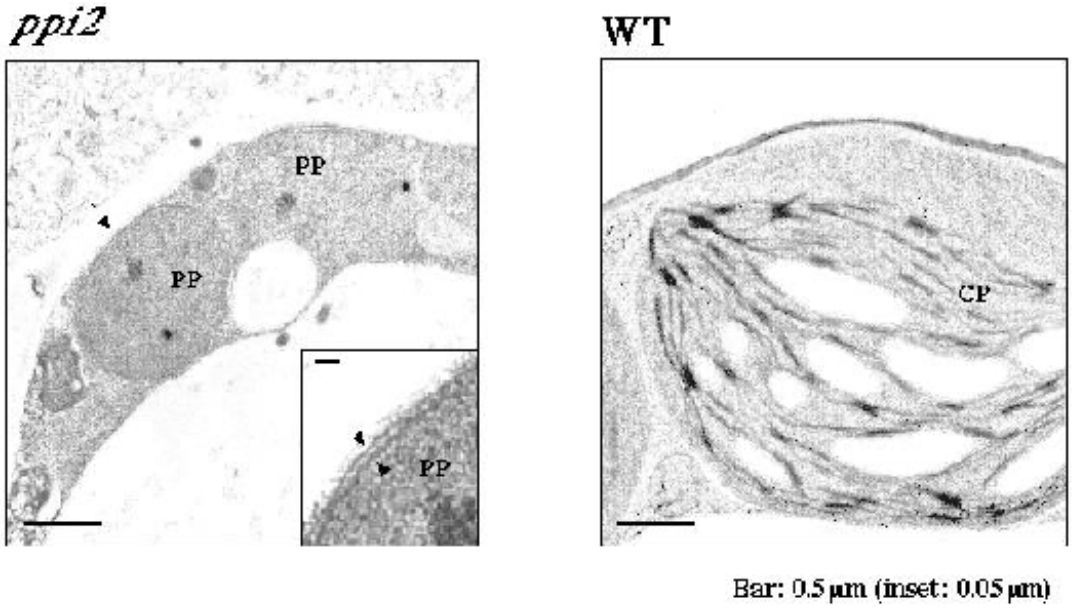

Figure Legend: Ultrastructure of ppi2 plastids. Transmission electron microscopy of plants grown on soil for 6 days in long-day conditions indicates that ppi2 plastids (ppi2) remain as undifferentiated proplastids (PP) compared to the chloroplasts (CP) present in wild-type (WT) cells. The lack of thylakoid membranes and starch granules in ppi2 plastids is indicative of a chloroplast biogenetic defect. Scale bar, 0.5?m. Inset, the double membrane envelope of the ppi2 proplastids; scale bar, 0.05 ?m.

TWO PROCESSING SITES AND ENZYMES INVOLVED IN CHLOROPLAST IMPORT OF THE SMALL SUBUNIT PRECURSOR FOR RUBISCO FROM *CHLAMYDOMONAS REINHARDTII*

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The small subunit (SS) of ribulose-1,5-bisphosphate carboxylase (RuBisCO) of plants and green algae is synthesised as precursor protein (pSS) in the cytoplasm and imported into the chloroplast where it is processed to the mature protein SS. In *Chlamydomonas reinhardtii* pSS consists of 185 amino acids of which the N-terminal 45 amino acids form the transit sequence and the remaining 140 amino acids the mature protein SS (Fig. 1).

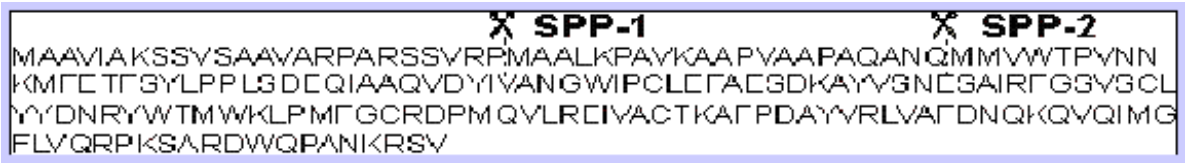


Figure 1: **Amino acid sequence of the precursor of the small subunit of RuBisCO (pSS) of *Chlamydomonas***. The two processing sites for the peptidases SPP-1 and SPP-2 are indicated.

Processing can be studied in cell free experiments by incubation of *in vitro* synthesised, radioactively labelled pSS with stromal proteins. Gelfiltration of stroma from *Chlamydomonas reinhardtii*, but not from higher plants, demonstrated the existence of different stromal processing peptidases (SPP), among them SPP-1 and SPP-2 [1,2] The fractions containing SPP-2 cleaved the precursor pSS to the mature protein SS, while the peptidase SPP-1 hydrolysed pSS in the transit sequence to an intermediate sized protein (iSS). These *in vitro* results were confirmed *in organello* by import of highly [^{35}S]met-labelled pSS into isolated chloroplasts. Such experiments resulted in three distinct radioactive bands on a polyacrylamide gel, namely pSS (21 kDa), the intermediate band iSS (19 kDa), and SS (17 kDa) [3]. Interestingly, iSS was shown to be located in the intermembrane space of the chloroplast envelope which might be a hint towards a two-step import into chloroplasts.

To verify this hypothesis we studied *in vitro* and *in organello* processing of pSS bearing mutations at the processing sites that are no longer recognised by the enzymes SPP-1 and SPP-2. We introduced mutations into a plasmid containing the cDNA encoding pSSHis, i.e. pSS with a C-terminal hexahistidyl-tail. Thereby, in the protein the following amino acids were exchanged:

SPP-1 site: **pSSM1PMHis**-cDNA: **Pro**-24–**Met**-25 changed to **Ile**-24–**Trp**-25

SPP-2 site: **pSSM2MHis**-cDNA: **Met**-46 changed to **His**-46

pSSM2QMHis-cDNA: **Gln**-45–**Met**-46 changed to **Ile**-45–**Trp**-46

A new restriction site for the enzyme *Nde*I facilitated detection of the mutations. From all cDNAs radioacitvely labelled mutated pSSHis proteins were synthesised and incubated with fractions enriched in peptidases SPP-1 or SPP-2 (Fig. 2).

Fig. 2 shows that wild type pSSHis was processed by SPP-1 and SPP-2 to iSSHis and SSHis, respectively, while pSSM1PMHis was efficently processed by SPP-2, but not by SPP-1. In contrast, the protein pSSM2MHis was cut by SPP-1 and by SPP-2 to the intermediate and mature proteins, respectively. pSSM2QMHis containing the double mutation in the SPP-2 processing site was cleaved by SPP-1, but not by SPP-2. Wherever such *in vivo* processing took place quantification of the bands revealed a comparable efficiency in processing of mutant and wild type pSS, conferming that *in vitro* the two enzymes act independently

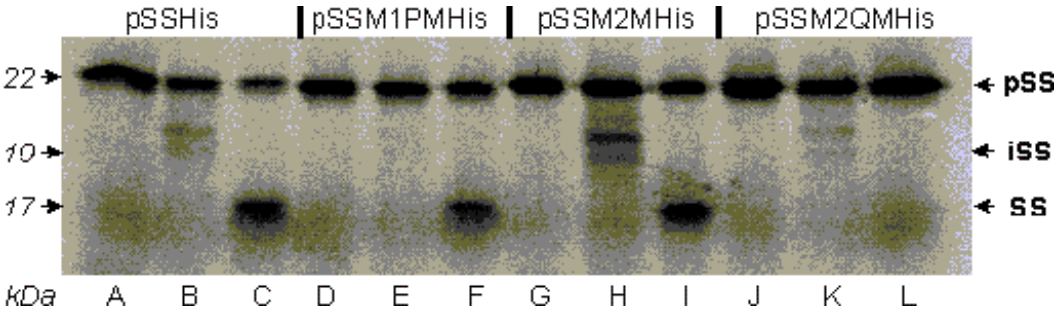
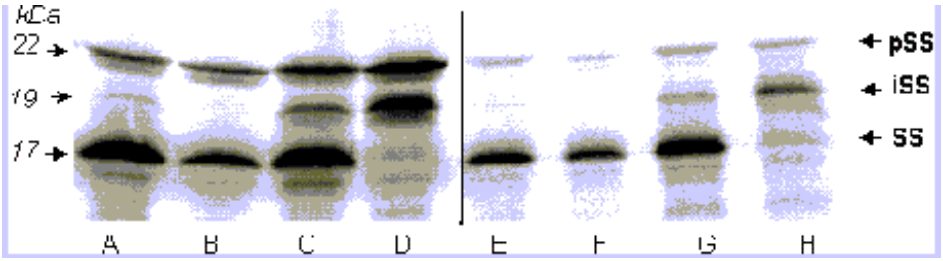


Figure 2: **Autoradiogram of an electropherogram of different mutated pSSHis proteins after *in vitro* processing with SPP-1 and SPP-2** For each protein the left lane (A,D,G,J) shows unprocessed protein, the middle lane (B,E,H,K) the protein incubated with SPP-1, the right lane (C,F,I,L) with SPP-2. The double band in place of iSS might be due to high salt concentration in the SPP-1 fraction which for this experiment was isolated by ion-exchange chromatography; when isolated by gelfiltration such doublet was not visible.

The three mutated proteins were subjected also to import into isolated chloroplasts (Fig. 3). When wild type pSSHis was imported (lane A) pSSHis, SSHis, and a very weak iSSHis band could be detected on a polyacrylamide gel. In contrast, import of the protein pSSM1PMHis (laneB) yielded only pSSHis and SSHis, but no iSSHis band. Import of pSSM2MHis (lane C) led to the formation of pSSHis, SSHis and iSSHis. Contrary to the *in vitro* results, the latter was of much higher intensity than upon import of wild type protein. Import of pSSM2QMHis (lane D) resulted in only two bands pSSHis and iSSHis, with almost no SSHis. Here also, iSSHis was significantly accumulated in comparison to wild type protein import. When after import pSS remaining adsorbed at the chloroplast envelope was removed by thermolysine (lanes E-H) similar results were obtained, except for the absence of the pSSHis bands.

Obviously, even when normal processing is prevented by a mutation, pSSHis will not accumulate within the chloroplast, but is rapidly processed at the other still intact site.

Figure 3: **Autoradiogram of an import of the different mutated pSSHis proteins into isolated chloro-plasts, either without (lanes A-D) or with (lanes E-H) an additional thermolysine treatment**. Lanes **A** and **E** show the import of wild type pSSHis protein, lanes **B** and **F** of mutated pSSM1PMHis protein, lanes **C** and **G** of mutated pSSM2MHis protein and lanes **D** and **H** of mutated pSSM2QMHis protein.



As after import pSSM2QMHis is processed to iSSHis but not to SS, while pSSM1PMHis formed no iSSHis but the mature SS, also within the chloroplast the two processing events seem to work independently. Nevertheless, since import of pSSM2MHis, which is processed by both enzymes, resulted in accumulation of iSSHis as compared to wild type protein import, some kind of interaction between the two processing events will exist. Partial inhibition of processing at the SPP-2 site might slow down the formation of SS from pSSHis, but also from iSSHis whereby this intermediate accumulates. Overall, the results suggest that in *Chlamydomonas* a two-step processing of pSS occurs, however, the first step is not obligate, but rather facultative.

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IS RUBISCO ACTIVASE A TEMPERATURE SENSOR IN PLANTS?

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After a dark phase, rubisco (EC 4.1.1.39) is present in an inactive form. Sugar phosphates are tightly bound to the active site and must be removed during the activation process (Salvucci 1993, Salvucci et al. 1985). Rubisco can be activated enzymatically in an ATP-dependent reaction catalized by rubisco activase or non-enzymatically at elevated CO_2 concentrations (Salvucci et al. 1985, Portis 1992, Portis et al. 1986). At ambient CO_2, Rubisco activase enhances the dissociation of sugar phosphates from rubisco and faciliates the carbamylation of an active-site lysine residue most likely by inducing specific conformational changes in rubisco that affect the binding affinity for sugar phosphates (Salvucci 1993, Salvucci and Ogren 1996). Since at ambient CO_2 concentration the acitvity of rubisco and as a consequence also of the Calvin cylce depends on rubisco activation by rubisco activase, the latter enzyme must be considered as a key regulatory factor in photosynthesis. Furthermore, rubisco activase is often present under two forms deriving from alternative splicing of pre-mRNA (Crafts-Brandner et al. 1997). The two forms of subunits differ in their properties, especially in their tolerance to elevated temperatures (Crafts-Brandner et al. 1997). Several lines of evidence suggest that rubisco activase is reversibly inactivated at moderatly elevated temperatures *in vivo* and that this inactivation down-regulates rubisco and the Calvin cycle:

At moderatly elevated temperature, the substrate of rubisco (ribulose 1,5-bis-phosphate) accumulates in intact leaf cells, while the level of the product (phosphoglycerate) declines (Law and Crafts-Brandner 1999, Weis 1981).

Rubisco activity measured rapidly after extraction according to a protocol avoiding activation during sample preparation is lower in wheat leaf segments incubated for 5 minutes at 30°C or at a higher temperature as compared to segments kept at 22.5°C (Feller et al. 1998). This effect is reversible, since the full enzyme activity was detected when samples were kept for 5 minutes at 30°C to 40°C and then for 15 minutes at 22.5°C. However, the activity is irreversibly lost when wheat leaf segments are kept for 5 minutes at 45°C. In cotton leaf discs, the reversible inactivation and the irreversible loss of rubisco activase is shifted to slightly higher temperatures than those observeded for wheat.

The carbon dioxide exchange rate of intact leaves is at elevated temperature closely associated with the activation status of rubisco but not with the transpiration rate (Law and Crafts-Brandner 1999). Leaf temperature was directly measured with a thermocouple. The differences between wheat and cotton were also evident in these investigations with intact plants.

Rubisco from wheat leaf segments kept at moderately elevated temperature (30-40°C) is fully activated *in vitro* to the level of control plants by incubating extracts with 10 mM $NaHCO_3$ for 10 minutes at 30°C (Feller et al. 1998). These results indicate that rubisco protein is still present and functional, although its activation status *in vivo* is affected considerably. This is consistent with findings of Eckardt and Portis (1997) indicating that rubisco is far more heat-stable than rubisco activase.

Nonphotochemical fluorescence quenching (qN) is increased at moderately elevated temperatures indicating that the thykaloid membrane is fully energized (Feller et al. 1998). The realtive initial fluorescence (F_0) and the maximal fluorescence (F_m) are not yet affected at 35°C in wheat. From the fluorescence data it can be concluded that the electron transport chain and the supply of the Clavin cycle with ATP and NADPH is still functional when the activation of rubisco becomes affected.

When the temperature in wheat leaf segments is increased to temperatures above 40°C, rubisco activase becomes insolubilized and partially cross-linked (Feller et al. 1998; Herrmann et al. 1998). The insolubilized enzyme is most likely precipitated and not membrane-associated, since rubisco activase remaines insoluble after dissolving the membranes with Triton X-100 (Feller et al. 1998). Besides the reversible inactivation at moderately elevated temperatures, an irreversible damage of rubisco activase must be considered at higher temperatures.

Rubisco activase is apparently very sensitive to elevated temperatures and is inactivated when other chloroplast enzymes are not yet affected. The different properties of the two rubisco activase polypeptides deriving from alternative splicing and the fact that the activation status of rubisco is influenced differently in wheat (less tolerant) and cotton (more tolerant) by elevated temperatures suggest that rubisco activase plays a key role in the heat tolerance of plant species.

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Poster C1

PURIFICATION OF A STROMAL PROCESSING PROTEASE FROM *CHLAMYDOMONAS REINHARDTII*

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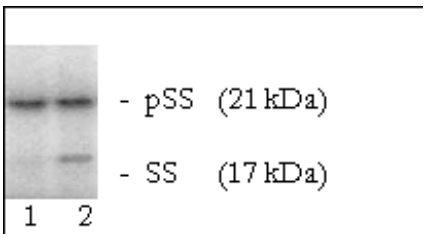
Most chloroplast proteins are synthesized in the cytoplasm as precursor proteins containing an N-terminal transitpeptide which mediates their import into the chloroplast. Immediately after the translocation, the transit sequence is cleaved off by a stromal processing protease (SPP) In contrast to higher plants where only one general processing enzyme has been postulated [1], *C. reinhardtii* contains at least 4 different highly specific SPPs [2]. SPP-2, one of these enzymes from *C. reinhardtii*, was characterized and purified in this project. It is located in the stroma and processes the precursor of the small subunit of ribulose-1,5-bisphosphate-carboxylase (pSS).

Knowing nothing about this enzyme but its proteolytic specificity, SPP-2 had to be detected indirectly by monitoring its biological activity, i.e. the processing of pSS to the mature protein (SS). For this purpose, the cloned cDNA of pSS was transcribed *in vitro* followed by translation in a wheat germ system in the presence of [^{35}S]-labelled methionine. After incubation of the radioactive pSS with a SPP-2 containing fraction, processing was detected by SDS-PAGE and autoradiography (Fig. 1). The presence of the protease resulted in proteolysis of pSS (21 kDa) and the appearance of a clearly visible band of SS (17 kDa). This activity assay was used throughout the whole experimental procedure to determine whether a putative SPP-2 containing fraction was positive for the protease.



SDS-PAGE and autoradiography of [^{35}S]-Met labelled pSS (lane 1) and proteolysis products pSS and SS (lane 2)

Starting with isolated chloroplasts of *C. reinhardtii* [3], soluble proteins were separated from membranes by freezing/thawing-cycles and centrifugation.

The further SPP-2 directed fractionation of stromal proteins comprised four major steps:

Q 15), resulting in a peak of highly enriched SPP-2. In a third step the active fractions were either electrophoretically separated by nondissociating PAGE (Fig. 2) or alternatively rechromatographed by gelfiltration (Sephadex 200) on a SMART system (Fig. 3). During both methods, the protease remained in its native form and activity could easily be assigned to the corresponding fractions (Fig. 2 and 3). Finally, in a fourth step the active fractions resulting from gelelectrophoresis or rechromatography were separated on SDS-PAGE, resulting in both cases in only three discrete bands of approximately 67, 65 and 52 kDa (Fig. 4). Aminoacid analysis showed identical composition of the two upper protein bands (67 and 65 kDa), suggesting that the small difference in size could be due to different posttranslational modifications of the otherwise same protein. Comparing the molecular weights of the three bands (67, 65 and 52 kDa) with the molecular weight of 65 kDa of the native enzyme, as determined by gelfiltration, no complexes or multimers are possible. For these reasons the biological activity of SPP-2 most probably has to be assigned to the upper double band on the SDS-PAGE.

Despite of a sufficient amount of the separated proteins we couldn't determine their N-terminal amino acid sequences so far, indicating blocked N-termini. Therefore preparation of higher amounts of SPP-2 is now in progress to allow fragmentation of the proteins and determination of internal sequences.

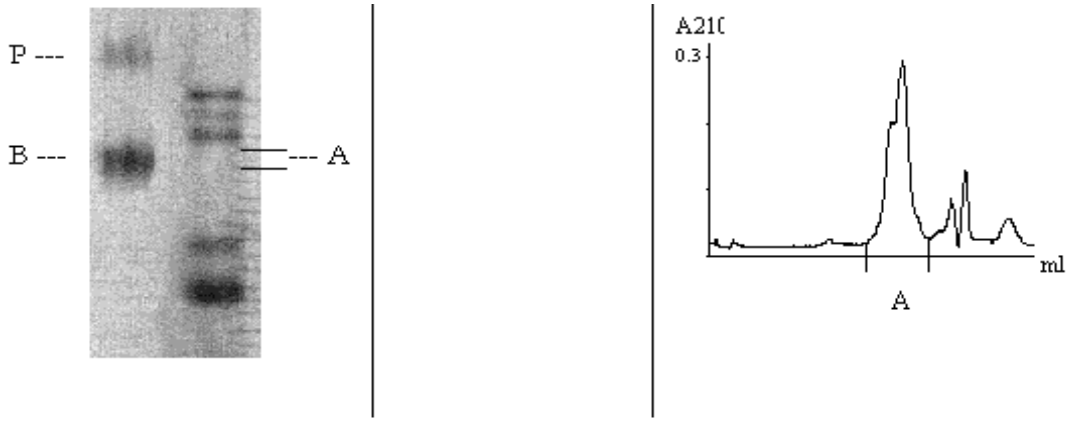


Figure 2: Native PAGE

Figure 3: Gelfiltration of the SPP-2 enriched fraction. Activity was detected in the fraction A.

Coomassie Blue staining of a nondissociating gel. Separation of phosphorylase b (P) and BSA (B) as marker proteins (lane M) and of the SPP-2 enriched fraction (lane F). Activity was detected at the position indicated by A.

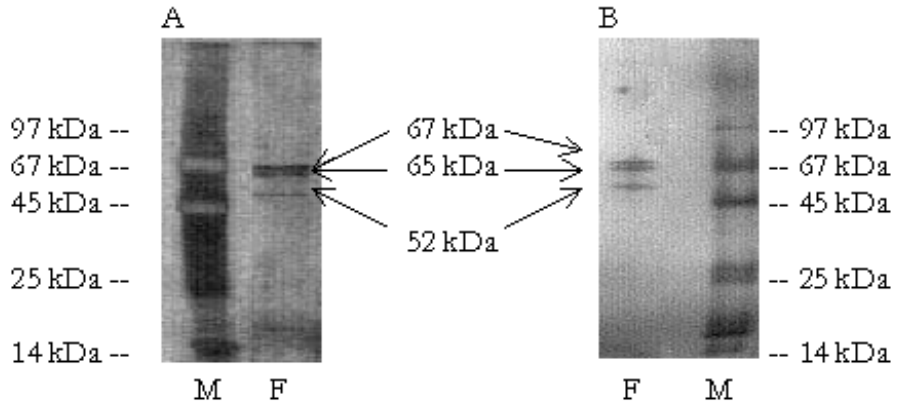


Figure 4: SDS-PAGE of purified SPP-2.

Silver stained SDS-poly-acrylamide gels, showing marker proteins (lane M) and SPP-2 enriched fractions (lane F) after native gel electophoresis (A), or gel filtration (B).

SPP-2 enriched fractions were used to determine the physico-chemical parameters of the processing enzyme. It worked best at the conditions of 25 °C and pH 7.8, had an isoelectric point of 3.9 and a molecular weight of approximately 65 kDa in its native form, corresponding with the double band (67 and 65 kDa) found on SDS-PAGE (Fig. 4). Using inhibitor studies, SPP-2 was assigned to the class of either serine proteases or metalloproteases [2].

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