Plant Physiol. (1997) 114: 715–722

A Plant Chloroplast Glutamyl Proteinase[1]

William A. Laing* and John T. Christeller

HortResearch, Private Bag 92169, Auckland, New Zealand (W.A.L.); and HortResearch, Private Bag 11030, Palmerston North, New Zealand (J.T.C.)

A glutamyl proteinase was partially purified from Percoll gradient-purified spinach (*Spinacia oleracea*) chloroplast preparations and appeared to be predominantly localized in the chloroplast stroma. The enzyme degraded casein, but of the 11 synthetic endopeptidase substrates tested, only benzyloxycarbonyl-leucine-leucine-glutamic acid-β-napthylamide was hydrolyzed at measurable rates. In addition, the enzyme cleaved the oxidized β-chain of insulin after a glutamic acid residue. There was no evidence that native ribulose-1,5-bisphosphate carboxylase/oxygenase was cleaved by this proteinase. The apparent K_m for benzyloxycarbonyl-leucine-leucine-glutamic acid-βNA at the pH optimum of 8.0 was about 1 mM. Cl^- ions were required for both activity and stability. Of the proteinase inhibitors covering all four classes of the endopeptidases, only 4-(2-aminoethyl)-benzenesulfonyl-fluoride HCl and L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone significantly inhibited the proteinase. The partially purified enzyme had a molecular weight of about 350,000 to 380,000, based on size-exclusion chromatography. The enzyme has both similar and distinctive properties to those of the bacterial glutamyl proteinases. To our knowledge, this is the first description of a plant glutamyl proteinase found predominantly or exclusively in the chloroplast.

Proteinases are important during protein breakdown in senescence-regulated protein turnover during normal metabolism, protein remobilization during development, and protein degradation during stress responses (Ryan and Walker-Simmons, 1981; Vierstra, 1993; Callis, 1995). In addition, proteinases are involved in the processing of proteins during their synthesis and transport within the cell (Lord and Robinson, 1986).

The chloroplast is a complex organelle containing its own partial genome (Taylor, 1989; Sugiura, 1992) and protein synthesis machinery (Gillham et al., 1994), as well as the capacity to degrade proteins (Dalling and Nettleton, 1986). The chloroplast has proven to be a source of several different proteinases, some of which have been isolated and partially characterized. Chloroplast proteinases include both soluble stromal and membrane-located enzymes. Some of the thylakoid-located proteinases described to date include enzymes that process protein presequences as well as others that degrade specific targets. Examples of the latter include three thylakoid-bound enzymes: a prolyl endoproteinase from spinach (*Spinacia oleracea*) that degrades the 18-kD protein from PSII (Kuwabara, 1992); another that degrades the Chl *a*/*b*-binding protein (Hoober and Hughes, 1992); and the membrane-bound NADPH-protochlorophyllide oxidoreductase proteinase of barley (*Hordeum vulgare*; Hauser et al., 1984). Examples of processing proteinases include one that processes the thylakoid D1 protein (Bowyer et al., 1992) and another that processes plastocyanin (Hageman et al., 1986). Other thylakoid proteinases have been characterized, but have yet to be ascribed a specific function (e.g. Kuwabara and Hashimoto, 1990). In addition, several soluble chloroplast proteinases have been described, including a stromal metalloproteinase that degrades Rubisco (Bushnell et al., 1993), metallo- and Ser-proteinases (Musgrove et al., 1989), a Cys proteinase (Liu and Jagendorf, 1986), and a soluble, metallo precursor-processing proteinase (Robinson and Ellis, 1984). A range of aminopeptidases have also been described from chloroplasts (Waters et al., 1982; Liu and Jagendorf, 1986; Thayer et al., 1988).

There have been reports of ATP-dependent proteolysis in chloroplasts (Liu and Jagendorf, 1984; Malek et al., 1984). Subsequently, a gene for the ATP-regulated proteinase homologous to the Ti proteinase (the *Escherichia coli* ClpP gene product) has been shown to be encoded by the chloroplast genome (Gray et al., 1990; Weglohner et al., 1992; Moore and Keegstra, 1993; Clarke et al., 1994). There has also been a recent report of the presence of the Ti protein in the chloroplast (Shankin et al., 1995).

Prokaryotic glutamyl proteinases (e.g. the *Staphylococcus aureus* V8 proteinase widely used in protein sequencing studies; Birktoft and Breddam, 1994) cleave a peptide bond that usually follows a Glu and, to a much lesser extent, an Asp. They are Ser proteinases, belonging to family S2, and have only been reported in eubacteria (Rawlings and Barrett, 1994). They are resistant to inhibition by most proteinase inhibitors, with a pH optimum in the range of 7.0 to 9.0 (Birktoft and Breddam, 1994). To our knowledge, no such proteinase with a specificity toward glutamyl residues has been described from plants to date, except for the Z-LLE-βNA-hydrolyzing activity associated with the proteasome (Ozaki et al., 1992; W.A. Laing, unpublished observations). However, there have been reports of proteinase inhibitors that appear to target putative glutamyl proteinases in plants (e.g. Margossian et al., 1988; Fujita et al., 1993;

[1]This project was funded by the Foundation for Research, Science and Technology, New Zealand.

* Corresponding author; e-mail wlaing@hort.cri.nz; fax 64–9–815–4201.

Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl-fluoride HCl; AMC, 7-amido-4-methylcoumarin; βNA, β-napthylamide Boc, *t*-butyloxycarbonyl; Chl, chlorophyll; Succ, succinyl; Z-LLE-βNA, benzyloxycarbonyl-Leu-Leu-Glu-βNA.

Linthorst et al., 1993), although their in vivo function is unknown.

This paper describes a high-molecular-weight chloroplast glutamyl proteinase with a mildly alkaline pH optimum and a dependency on Cl^- ions for activity. This enzyme shares many of the characteristics of the bacterial glutamyl proteinase, although in other features it differs markedly.

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea* L.; hybrid number 7R, Yates New Zealand Ltd., Auckland, New Zealand) plants were grown hydroponically under fluorescent lights (Roughan, 1994). Synthetic peptide substrates were obtained from Bachem AG (Bubendorf, Switzerland) or Sigma, and fluorescent (Bodipy-labeled) casein was obtained from Molecular Probes (San Diego, CA). Other biochemicals were obtained from Sigma. Chromatography media were obtained from Pharmacia Biotech (Uppsala, Sweden) or, in the case of hydroxyapatite, from Bio-Rad, whereas the Miniprotean II and Prepcell electrophoresis systems from Bio-Rad were used for gel electrophoresis. Fluorescence was measured on a microwell-plate-reading fluorimeter (Fluroskan II 96, Labsystems, Hampshire, UK). Filtration equipment (Amicon, Beverly, MA) and 10,000 molecular weight cutoff membrane filters (Amicon) were used to concentrate samples.

Partial Purification of the Proteinase

All procedures were carried out at 4°C or lower. Intact chloroplasts were isolated and purified using the method described by Roughan (1994). This included centrifugation on a Percoll gradient, which results in highly purified chloroplasts. The chloroplasts were immediately frozen in liquid nitrogen and stored at −80°C until used. The frozen chloroplasts were thawed and diluted 3-fold into 25 mM potassium phosphate buffer, pH 7.5, with 20% (v/v) glycerol and 0.1% (v/v) 2-mercaptoethanol. All other buffers, unless specified, contained these concentrations of glycerol and 2-mercaptoethanol. The lysed chloroplast extract was centrifuged at 30,000*g* for 10 min, and the clear supernatant fluid was applied to a 10 × 2.5-cm hydroxyapatite column equilibrated with phosphate buffer. The column was washed with this buffer and enzyme activity eluted at 1 mL min^{-1} using a 25 to 250 mM linear phosphate gradient.

The two peaks of Z-LLE-βNA-hydrolyzing activity were collected and concentrated to about 1.5 mL using 10,000 molecular weight cutoff membrane, and the concentrated samples were applied separately to a G75 Sephadex column (50 × 1.25 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The column was eluted at 1 mL min^{-1} with the same buffer, and void-volume fractions able to hydrolyze Z-LLE-βNA were combined and applied to a 1-mL anion-exchange column (HiTrap Q, Pharmacie Biotech, Uppsala, Sweden). The column was washed with the Tris-HCl buffer and the proteins were eluted with a 0 to 0.25 M NaCl linear gradient at 1 mL min^{-1} in this buffer. Active fractions were concentrated as above, and applied to a 50 × 1.25 cm high-resolution gel-filtration column (Sephacryl S-300, Pharmacie Biotech) equilibrated with Tris buffer. This column was calibrated using a range of protein standards to allow calculation of the M_r of the Z-LLE-βNA-hydrolyzing activity. Active fractions were concentrated, frozen in liquid nitrogen, and stored at −80°C. Most of the work characterizing the proteinase was performed using this level of purity. However, to further purify the enzyme, it was subjected to preparative native PAGE at pH 8.8 (Hames and Rickwood, 1983) at 4°C using a Prepcell with 20% (v/v) glycerol present in the 5% polyacrylamide gel.

Assays

The Z-LLE-βNA (dissolved in DMSO as a 2.5 mM stock solution) and fluorescent-casein-hydrolyzing activities were measured using a microwell-format fluorometer with interference filter peak excitation and emission wavelengths at 340 and 405 nm, respectively, for Z-LLE-βNA, and 485 and 538 nm, respectively, for caseinase activity. Assays of blocked peptide substrates with AMC derivatives (also dissolved in DMSO) had excitation and emission wavelengths of 355 and 460 nm, respectively. Standard assays (e.g. assays of column fractions) contained 50 mM Tris-HCl, pH 8.0, with 0.1% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.25 mM Z-LLE-βNA substrate, and 10% (v/v) DMSO in a final concentration of 100 μL. These assays automatically contained about 30 mM Cl^-, since the Tris base was adjusted to pH 8.0 with HCl. Assays were initiated by adding the substrate. In inhibition studies the inhibitor and enzyme were incubated together for at least 10 min before the substrate was added to ensure that equilibrium between the proteinase and the inhibitor was established. At least six time points were read in any assay with the plate incubated at 30°C between readings, and the results were collected by a computer and analyzed using programs similar to those described by Christeller et al. (1990). Only those points that showed a linear time course were used to calculate the reaction rate. Except for the caseinase reaction, reaction rates were a linear function of the amount of enzyme. Fluorescence calibration curves were carried out using AMC and βNA standards under a range of experimental conditions, and rates of reaction in appropriate units were calculated.

The effect of assay pH was determined as above, except that the effect of buffer composition was checked in a preliminary experiment. Since all buffers used except borate gave a continuous set of curves, subsequent pH curves were determined using 1,3-bis (Tris[hydroxymethyl] -methylamino)propane. Fluorescence yield was measured in βNA standard solutions as a function of pH, and was found to be almost independent of pH. Background assays at each pH value without added enzyme showed that the substrate was stable at all pH values. Substrate response curves were obtained by adding different amounts of substrate dissolved in a constant added volume of DMSO to each reaction. Background activities were measured in the absence of enzyme.

Activities were measured in crude leaf (the centrifuged extract made immediately after the initial leaf homogenization during chloroplast preparation) and chloroplast stromal extracts by freezing the extracts in liquid nitrogen, and slowly thawing them twice before centrifugation at 12,000*g* for 10 min to remove membrane-bound pigments that quench fluorescence during assay. Assays were carried out as above over a range of extract amounts, and results were calculated where assays responded linearly with the amount of extract assayed. Rubisco (Laing and Christeller, 1976) and NADH-malic dehydrogenase (Ting, 1968) were also used as markers for chloroplast purity. Protein was measured using BSA as a standard (Bradford, 1976) and Chl was measured as described by Roughan (1994).

Substrate cleavage specificity was measured using the oxidized β-chain of insulin. Enzyme was incubated with insulin for up to 48 h in the standard reaction mixture at 25°C, and at intervals 200-μL aliquots were removed into 800 μL of 0.1% (v/v) trifluoroacetic acid in water and immediately frozen at −20°C. Cleaved fragments were separated from the intact insulin chain by HPLC using a 250 × 4.6 mm reverse-phase column (C8, Vydac, Hesperia, CA) and a 0 to 100% (v/v) acetonitrile gradient containing 0.1% (v/v) trifluoroacetic acid at 1 mL min^{-1}. Representative samples of the newly generated fragments were sequenced on a Procise sequencer (Applied Biosystems) for four cycles. Cleavage of Rubisco was measured by incubating the Prepcell-purified proteinase (0.26 nmol min^{-1} activity for up to 40 h) in the presence of excess Rubisco (obtained from the Prepcell native gel purification step) both in the absence and presence of 5 mM $MgCl_2$ and ATP. The reaction was stopped by adding SDS sample buffer, and accumulated samples were analyzed by SDS-PAGE and Coomassie blue staining of the gel. Controls were performed without added glutamyl proteinase.

Polyacrylamide Electrophoresis

Tricine-SDS-PAGE was carried out as described by Schägger and Von Jagow (1987), and native gels (Hames and Rickwood, 1983) were electrophoresed at pH 8.8 and 4°C with the addition of 20% (v/v) glycerol to the gel. Gels were stained using a silver-staining procedure, and the native gels were stained using an activity assay (Van Der Valk et al., 1989). This consisted of a 20-min wash of the gel (with shaking) after electrophoresis in the Tris-HCl buffer containing 10 mM $MgCl_2$. Then, 10 mL of fresh buffer containing 0.125 mM Z-LLE-βNA was added and the gel was incubated at 30°C for 15 to 30 min. The gel was then briefly washed with distilled water to remove excess substrate and 20 mL of 0.1% (w/v) fast garnet dye was added. The development of dark-red-stained bands on an orange background was followed and, when staining appeared complete, the gel was washed several times with water, photographed, and dried under a vacuum at 80°C.

Data Analysis

Data were fitted to untransformed theoretical equations using the SAS (SAS Institute, 1985) statistical package. Calculated parameters are presented as the value ± the asymptotic SE. Experiments were repeated two or three times, and either representative experiments are shown or the means of several experiments are presented as noted in the figures and tables.

RESULTS

Purity of Chloroplasts and Location of the Proteinase

Initial experiments with spinach leaves indicated that crude extracts were highly capable of hydrolyzing the synthetic peptide substrate Z-LLE-βNA at pH 8.0. These fluorescent assays were strongly quenched by the green plant extract and, thus, had to be carried out at low-extract concentrations to ensure a linear response of reaction rate to extract volume. This was facilitated by centrifugation of the extract to remove as much membrane-bound Chl as possible. Measurements of the hydrolysis of Z-LLE-βNA by extracts of Percoll gradient-purified spinach chloroplasts showed that high levels of this proteinase activity were also located in the chloroplast. Activity assays following native-PAGE separation of extracts of whole leaves and intact chloroplasts showed only one band of activity at the same position for both samples in the gel (Fig. 1, A and B), suggesting that if the proteinase was located in more than one site, then all forms were of a similar mass/charge ratio and mass. Sequential extractions of the chloroplasts and thylakoid membranes after breakage showed that over 90% of the chloroplast-located Z-LLE-βNA-hydrolyzing activity was soluble in the first extraction with Tris buffer.

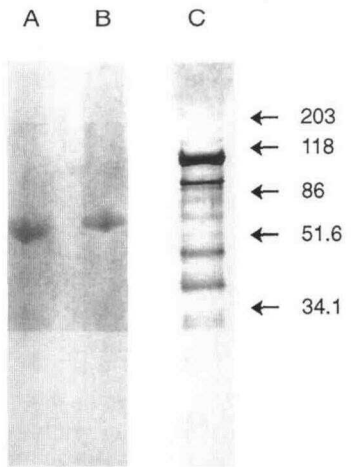

Figure 1. Native-PAGE resolution of the glutamyl proteinase. An extract of purified chloroplasts (originally containing 3.3 μg of Chl) was loaded in lane A and a whole-leaf extract (originally containing 13 μg of Chl) was loaded in lane B. Gels were electrophoresed at 4°C for 90 min, reequilibrated with standard buffer, and stained for Z-LLE-βNA-hydrolyzing activity, as described in the text. The gel was color-photographed and digitally scanned, and the red stain of the Z-LLE-βNA-hydrolyzing activity was selected from the orange background using image analysis. The selected image was converted to a gray scale for reproduction. In lane C, less than 0.5 μg of the 10-fold-concentrated, Prepcell-purified glutamyl proteinase was separated by SDS-PAGE and silver-stained. Protein markers are enumerated to the right of lane C (values in kilodaltons).

To determine what proportion of the proteinase was located in the chloroplast, we assayed whole-leaf extracts and the purified chloroplasts made from this extract, and expressed the results on a Chl basis. If activity was predominantly located in the chloroplast, then we would expect a similar activity per unit of Chl in whole-leaf and chloroplast extracts. This was indeed the case, with the ratio of Z-LLE-βNA-hydrolyzing activity per milligram of Chl of chloroplast extract to whole-leaf extract being 0.80, implying four times as much Z-LLE-βNA-hydrolyzing activity in the chloroplast as in the rest of the cell. Two marker enzymes were also measured, NADH-malic dehydrogenase (cytoplasm, peroxisome, and mitochondrion [Ting, 1968]) and Rubisco (chloroplast). The purified chloroplasts had about 1% of the total leaf malic dehydrogenase activity on a Chl basis. On the other hand, the levels of Rubisco activity were similar in the two extracts on a Chl basis (1.2–1.0). These results clearly show that the isolated chloroplasts were highly pure, and that at least two-thirds and possibly all of the Z-LLE-βNA-hydrolyzing activity is located in the chloroplast.

Enzyme Purification

We partially purified this proteinase from Percoll-purified chloroplasts using a combination of hydroxyapatite chromatography, anion-exchange chromatography, and Sephacryl S-300 gel filtration. The column-chromatography profiles during these three steps are shown in Figure 2, A to C, and a summary of the yields is given in Table I. In addition, an example of purification using the Prepcell is shown. During the initial hydroxyapatite chromatography, the enzyme activity divided into two peaks (Fig. 2A). These two samples were subsequently purified separately and are referred to as peak 1 and peak 2 Z-LLE-βNA-hydrolyzing activity. Both fractions showed very similar behavior in the following chromatographic steps and in their properties, and in the remainder of Figure 2 (B–D) only one of the two preparations is shown.

The active hydroxyapatite eluates were concentrated separately using a 10,000 M_r cut-off membrane and desalted on a Sephadex G75 column (in which they eluted in the void volume [data not shown]) and then further purified on an anion-exchange column (Fig. 2B). The fractions with the highest activity were then concentrated and passed through a Sephacryl S-300 gel-filtration column (Fig. 2C). At this stage the highest-activity eluates had several bands of protein present following SDS-PAGE, although only one band of activity was present on a native PAGE system stained with Z-LLE-βNA (data not shown). This enriched but partially purified preparation was used for most of our studies because of the low amounts of protein available. However, a further stage of purification was achieved using a preparative native electrophoresis system. A single band of activity eluted from the preparative gel, but the protein amounts were too low to yield accurate values. The major contaminating protein eluting from the gel was shown to be Rubisco. Following preparative electrophoresis, there were one major and three minor bands of protein on a silver-stained SDS-PAGE gel (Fig. 1C). The major band had a M_r of 112,000 and was the only band to stain with Coomassie blue. There was also a much weaker band of 90 kD and two minor bands between 34 and 51 kD. We do not know which, if any, of these bands was the glutamyl proteinase. Size-exclusion chromatography during the final, routine stage of purification (Fig. 2C) and after the Prepcell stage of purification (data not shown) on a calibrated Sephacryl S-300 column showed that the enzyme had a native M_r of 350,000 to 380,000.

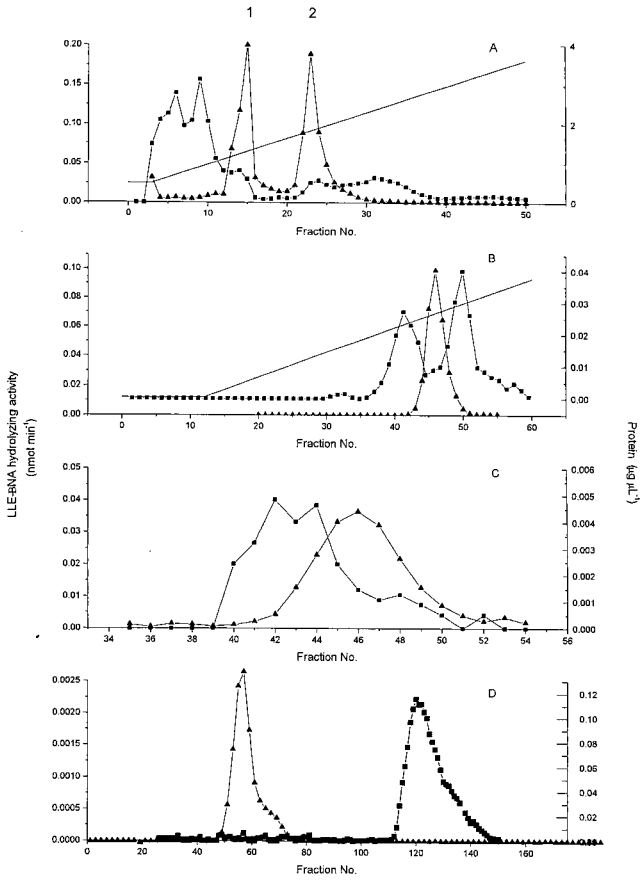

Figure 2. Chromatographic purification of chloroplast proteinases. A, Hydroxyapatite chromatography of the clarified chloroplast extract. The linear gradient of Pi is shown as a straight line starting at 25 mM and continuing to 160 mM. B, Anion-exchange chromatography on a HiTrap Q column of the concentrated combined fractions of peak 2 from A. The linear gradient of NaCl is shown as a straight line from 0 to 170 mM. C, Size-exclusion chromatography on a Sephacryl S-300 column of the concentrated peak 2 active fractions from B. D, Representative Prepcell purification of the enzyme from the Sephacryl S-300 column. In all panels the squares represent the protein amount, the triangles represent the Z-LLE-βNA-hydrolyzing activity, and the solid lines represent the concentration of eluting salt.

Properties of the Enzyme

There were two distinct forms of the Z-LLE-βNA-hydrolyzing activity on the hydroxyapatite column (Fig. 2A). However, in subsequent characterization of these enzyme forms, they always showed essentially identical be-

Table I. *Purification of Z-LLE-βNA-hydrolyzing activity from purified spinach chloroplasts*

All assays were carried out in 50 mM Tris-HCl buffer at pH 8.0 with no added Cl^- ions. Chloroplasts contain about 40% of the soluble protein in the cell. Consequently, the specific activity of the Z-LLE-βNA-hydrolyzing activity on a whole-leaf basis would be about 0.12 nmol min^{-1} mg^{-1} (crude protein). The data in this table are representative of two major preparations, and similar fractionation results were obtained in a preparation made from leaves.

Fraction	Protein	Activity	Specific Activity	Purification
	mg	*nmol* min^{-1}	*nmol* $min^{-1}mg^{-1}$ *protein*	*-fold*
Chloroplasts	87	22.7	0.26	1
Hydroxyapatite				
Peak 1	5.4	11.9	2.2	8
Peak 2	4.4	12.8	2.9	11
HiTrap Q				
Peak 1	0.49	4.8	9.8	38
Peak 2	0.15	3.7	24.7	95
Sephacryl/ S-300				
Peak 1	0.12	2.8	23.3	90
Peak 2	0.03	3.7	123	473

havior and properties. Consequently, for clarity, in some cases only one form is shown in the figures. The rate of Z-LLE-βNA hydrolysis depended upon the salt concentration in the assay. Time courses of Z-LLE-βNA hydrolysis were linear for at least the first 50 min at a high salt concentration (above 20 mM NaCl), whereas the rate declined with time at low salt concentrations (Fig. 3A). Consequently, the response of Z-LLE-βNA hydrolysis to salt concentration depended on the extent of the time course, showing a much greater response to salt when rates were calculated later in the time course (Fig. 3B). We have not yet established the specific ion(s) responsible for this salt effect, although NaCl and $MgCl_2$ are almost equally effective in stimulating the enzyme when measured at the same Cl^- concentration (Fig. 3B).

We tested the partially purified Z-LLE-βNA-hydrolyzing activity at pH 8.0 against a range of potential substrates. Rates of reaction with all substrates were measured by fluorescence assays, which are about 100 times more sensitive than the corresponding spectrophotometric or radiometric assays. The substrates tested included (at 0.25 mM): Succ-Ala-Ala-Phe-AMC (chymotrypsin-like), Succ-Phe-Leu-Phe-4-methoxy-βNA (chymotrypsin-like and a substrate of the *Escherichia coli* proteinase La), Boc-Phe-Ser-Arg-AMC (trypsin-like), Z-Gly-Gly-Arg-AMC (trypsin-like), Succ-Gly-Pro-Leu-Gly-Pro-AMC (prolyl endopeptidase substrate), Boc-Gly-Arg-Arg-AMC (trypsin-like), Succ-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like), Succ-Leu-Tyr-AMC (a substrate for the *E. coli* proteinase Ti), Succ-Tyr-Val-Ala-Asp-AMC (another minor substrate of the V8 glutamyl proteinase), and casein (general proteinase). None of these artificial substrates was hydrolyzed at rates faster than 0.1% of the rate of Z-LLE-βNA (the limit of sensitivity of the assay; data not shown). In addition, separate assays established that there were no benzoyl-Arg-4-nitroanilide (trypsin-like)- or benzoyl-Tyr-4-nitroanilide (chymotrypsin-like)-hydrolyzing activity present in spinach chloroplasts.

When the partially purified enzyme (after the Prepcell step) was incubated with insulin, two new peptide peaks on the HPLC trace were produced. The size of these peaks increased over 44 h of incubation, with 30% hydrolysis of the insulin occurring after 16 h and 68% hydrolysis after 44 h. Complete recovery of the sum of the amount of insulin and the two cleavage fragments was maintained throughout the time course. One had the sequence FVNQ, the initial N-terminal sequence of the 30-amino acid protein, and the other had the sequence ALYL, corresponding to residue numbers 14 to 17, immediately after a glutamyl residue. No other new peaks were observed. The enzyme did not cleave oxidized RNase A and the glutamyl proteinase did not cleave native spinach Rubisco in the presence or absence of ATP and $MgCl_2$, as shown by the fact that neither the large nor small subunits were altered in size or amount, as shown by SDS-PAGE (data not shown).

The response to the concentration of the substrate Z-LLE-βNA was measured at pH 8.5. Activity increased with substrate concentration to 1.0 mM and decreased slightly thereafter (Fig. 4). The apparent K_m (Z-LLE-βNA) values for peaks 1 and 2 (as identified from the hydroxyapatite column) were 1.04 (±0.04) and 1.17 (±0.22) mM, respectively. There appeared to be substrate inhibition at the highest concentrations, as determined from the deviation of the data points from the fitted hyperbola, which may reflect fluorescence quenching at high substrate concentrations. All other assays were done at 0.25 mM Z-LLE-βNA (or as noted), which is close to the linear part of the substrate response curve.

The pH response curve for the Z-LLE-βNA-hydrolyzing activity in 1,3-bis(Tris[hydroxymethyl]-methylamino)propane buffer adjusted between pH 6.5 and 10.5 was determined. In preliminary experiments it was established that the response to ionic strength was unaffected by pH (data not shown) and, thus, all assays were done at a constant saturating Cl^- concentration of 33 mM. The pH optimum under the constant substrate concentration used in this assay (0.14 mM) was close to pH 8.0 (data not shown). In a second experiment the effect of pH near the pH optimum on the apparent K_m(Z-LLE-βNA) and V_{max} was mea-

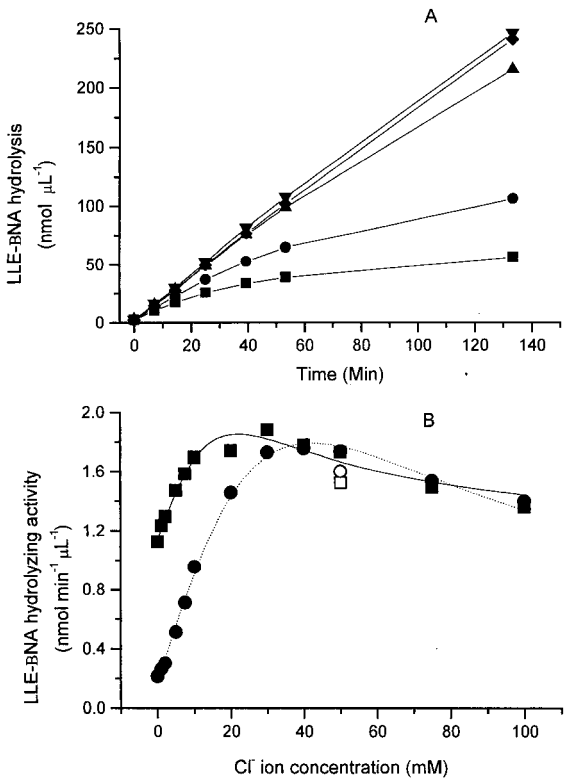

Figure 3. The response of Z-LLE-βNA-hydrolyzing activity to Cl^- ions. A, Time courses of activity at Cl^- concentrations of 0 (■), 5 (●), 20 (▲), 30 (▼), and 50 (◆) mM. B, Enzyme activity at the calculated initial rate (0–8 min [■]) and the final rate (47–133 min [●]), as affected by NaCl concentration. The open symbols (□ and ○) represent the corresponding rates carried out using $MgCl_2$. Peak 2 enzyme after the Sephacryl S-300 column was desalted using a HiTrap desalting column equilibrated with 50 mM Bicine, pH 8.5, and was essentially Cl^- free. Assays were carried out in the same buffer, with the indicated added [Cl^-], at a Z-LLE-βNA concentration of 0.25 mM.

sured (Table II). With increasing pH, the K_m rose to a maximum of about 1 mM Z-LLE-βNA and the V_{max} increased to a maximum at pH 8.75 at saturating substrate concentrations and then declined.

A range of proteinase inhibitors was evaluated against the Z-LLE-βNA-hydrolyzing activity to classify this proteinase. A range of inhibitors specific to Cys proteinases (E64 and cystatin), Ser proteinases (bovine pancreatic trypsin inhibitor, potato inhibitor I and II, soybean trypsin inhibitor, and AEBSF), both Cys and Ser proteinases (PMSF, leupeptin, chymostatin, and L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone), metalloproteinases (1, 10-phenanthroline-2, 2′-bipyrridyl, EDTA, and EGTA), and Asp proteinases (pepstatin), as well as a nonspecific inhibitor α-2 macroglobulin had little or no effect on the Z-LLE-βNA-hydrolyzing activity (data not shown). L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone, a Cys and Ser proteinase inhibitor, was the most effective (e.g. 90% inhibition at 2.6 mM), whereas at very high concentrations the Ser proteinase inhibitor AEBSF was also an effective inhibitor of the Z-LLE-βNA-hydrolyzing activity, with one-half-maximal inhibitory activity at about 8 mM AEBSF (data not shown).

DISCUSSION

To our knowledge, this is the first report of a proteinase showing strong specificity toward glutamyl residues in plants. Proteinases with a similar preference for cleaving after Glu residues have been reported from a range of bacteria, including *Staphylococcus aureus*, *Actinomyces* species, and various *Streptomyces* species, but they have not been previously reported in eukaryotes. The bacterial groups of glutamyl proteinases are characterized as Ser proteinases, with a preference for cleaving the peptide bond following a Glu or, to a much lesser extent, an Asp residue, and are generally not sensitive to inhibition by a range of proteinase inhibitors (Birktoft and Breddam, 1994). The proteinase reported in this paper showed no tendency to cleave after a range of hydrophobic or basic residues, or after Pro. Similarly, it was resistant to inhibition by a wide range of proteinase inhibitors. Although glutamyl proteinases have not been reported from plants, genes for glutamyl proteinase inhibitors have been shown to be expressed in ripening fruit (Margossian et al., 1988), in tobacco cell tumors (Fujita et al., 1993), and under stress (Linthorst et al., 1993). However, the possible function of these proteinase inhibitors, such as to inhibit endogenous proteinases or to protect the fruit from microbial disease, is unknown.

This proteinase from spinach-leaf chloroplasts is similar to the archetypal glutamyl-cleaving V8 proteinase from *S. aureus* in several of its properties. The chloroplast enzyme cleaves the substrate Z-LLE-βNA, which is diagnostic of this type of proteinase, but does not cleave significantly after an aspartic residue. The V8 proteinase also has about 1/1,000 the activity after an Asp residue compared with a Glu residue (Birktoft and Breddam, 1994). This stromal proteinase definitely has no activity for a range of other typical Ser proteinase substrates. In addition, it cleaved insulin after one of two residues present in the β-chain, again confirming its substrate specificity. The pH optimum of around 8.0 is typical of V8 proteinases, as is the lack of

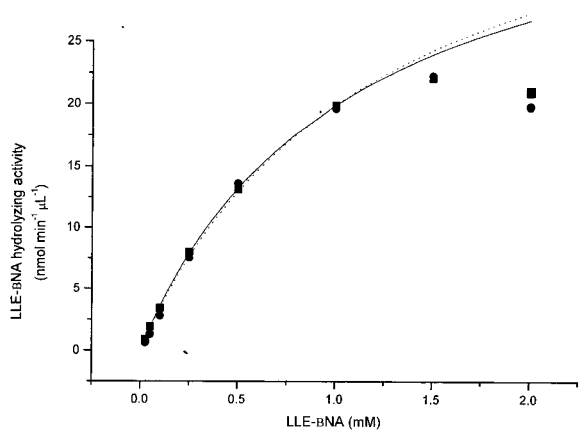

Figure 4. Substrate response curves of peaks 1 and 2 proteinases after the Sephacryl S-300 column step. Assays were carried out in 50 mM Bicine buffer at pH 8.5 and 32 mM Cl^-. The hyperbolic response curve was fitted to all points except the two highest Z-LLE-βNA concentrations. ●, Peak 1 enzyme; ■, peak 2 enzyme.

Table II. *Calculated V_{max} and K_m (Z-LLE-βNA) values as a function of pH for the peak 2 proteinase*

Reactions were carried out in 50 mM Bicine buffer at the adjusted pH with 32 mM NaCl. Values are means ± asymptotic SE.

Assay pH	K_m	V_{max}
	mM	nmol min^{-1} μL^{-1} enzyme
7.69	0.39 ± 0.05	2.64 ± 0.17
8.47	0.76 ± 0.04	4.31 ± 0.14
8.54	1.2 ± 0.22	4.33 ± 0.51
8.75	0.94 ± 0.04	4.54 ± 0.13
9.62	1.0 ± 0.07	1.31 ± 0.06

response to most proteinase inhibitors (except L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone and very high concentrations of AEBSF). However, this plant enzyme appears to be considerably different in its apparent native M_r (350,000–380,000) from the bacterial enzymes, which have a M_r less than 30,000 (Birktoft and Breddam, 1994). In addition, the stimulation of proteinase activity by Cl^- ions has not been previously reported as a property of glutamyl proteinases, and in several cases monovalent cations have been reported to be inhibitory (Birktoft and Breddam, 1994). Because the same rates were obtained at equimolar concentrations of Cl^- in the presence of differing levels of the magnesium and sodium counterions, we favor the possibility that we have observed an anion response, in this case to Cl^-. The rates with no exogenous Cl^- presumably reflect the presence of low levels of contaminating Cl^- or some other activating anion in the enzyme extraction buffer and, therefore, Cl^- may act as an activator, stabilizing the enzyme in an active form at high concentrations of Cl^- (Fig. 3A). Other chloroplast proteinases have been reported to be stimulated by Mg^{2+} (e.g. Anastassiou and Argyroudi-Akoyunoglou, 1995), but since these stimulatory effects are generally shown at Mg^{2+} concentrations of less than 1 mM, compared with greater than 20 mM for Cl^-, it is unlikely that they reflect a response to Cl^-.

Whether this chloroplast enzyme is related in any way to the glutamyl proteinases found in bacteria will have to await the generation of primary structure information. However, the location of this soluble enzyme in the chloroplast may reflect the prokaryotic origin of this organelle. Another example of a bacterial-type proteolytic enzyme also located in the chloroplast is the ClpP/ClpA-ATP-dependent proteinase (e.g. Gray et al., 1990).

There have been other reports of chloroplast proteolytic activities that have properties with some similarities to the Z-LLE-βNA-hydrolyzing activity reported in this paper. For example, Bushnell et al. (1993) reported the presence of a metallo-endoproteinase isolated from pea chloroplasts that apparently was contaminated with a Ser proteinase sensitive to AEBSF. However, these authors did not characterize this activity further, and whether this latter enzyme is the glutamyl proteinase described in our work is not known. Otto and Feierabend (1994) reported an inhibitor-insensitive proteinase that degraded Rubisco at pH 8.0, which had some association with the chloroplast membranes. However, our enzyme appeared completely soluble in dilute phosphate buffer and did not hydrolyze native Rubisco. Neither of these reported activities have been characterized as to their substrate specificity. In this paper we describe a new glutamyl proteinase, which is found predominantly or exclusively in the chloroplast stroma. This enzyme has several features in common with bacterial glutamyl proteinases, although in other respects it appears quite different.

Received December 26, 1996; accepted March 12, 1997.

LITERATURE CITED

Anastassiou R, Argyroudi-Akoyunoglou JH (1995) Thylakoid bound proteolytic activity against LHC II apoprotein in bean. Photosynthesis Res **43:** 241–250

Birktoft JJ, Breddam K (1994) Glutamyl endoproteinases. Methods Enzymol **244:** 114–126

Bowyer JR, Packer JCL, McCormick BA, Whitelegge JP, Robinson C, Taylor MA (1992) Carboxyl-terminal processing of the D1 protein and photoactivation of water-splitting in photosystem II: partial purification and characterization of the processing enzyme from *Scenedesmus obliquus* and *Pisum sativum*. J Biol Chem **267:** 5424–5433

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bushnell TP, Bushnell D, Jagendorf AT (1993) A purified zinc protease of pea chloroplasts, EP1, degrades the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol **103:** 585–591

Callis J (1995) Regulation of protein degradation. Plant Cell **7:** 845–857

Christeller JT, Laing WA, Shaw BD, Burgess EP (1990) Characterization and partial purification of the digestive proteases of the black field cricket *Teleogryllus commodus* (Walker): elastase is a major component. Insect Biochem **20:** 165–172

Clarke AK, Gustafsson P, Lidholm A (1994) Identification and expression of the chloroplast clpP gene in the conifer *Pinus contorta*. Plant Mol Biol **26:** 851–862

Dalling MJ, Nettleton AM (1986) Chloroplast senescence and proteolytic enzymes. *In* MJ Dalling, ed, Plant Proteolytic Enzymes, Vol 2. CRC Press, Boca Raton, FL, pp 25–153

Fujita T, Kouchi H, Ichikawa T, Syono K (1993) Isolation and characterization of a cDNA that encodes a novel proteinase inhibitor I from a tobacco genetic tumor, Plant Cell Physiol **34:** 137–142

Gillham NW, Boynton JE, Hauser CR (1994) Translational regulation of gene expression in chloroplasts and mitochondria. Annu Rev Genet **28:** 71–93

Gray JC, Hird SM, Dyer TA (1990) Nucleotide sequence of a wheat chloroplast gene encoding the proteolytic subunit of an ATP-dependent protease. Plant Mol Biol **15:** 947–950

Hageman J, Robinson C, Smeekens S, Weisbeek P (1986) A thylakoid processing protease is required for complete maturation of the lumen protein plastocyanin. Nature **324:** 567–569

Hames BD, Rickwood D (1983) Gel electrophoresis of proteins: a practical approach. IRL Press, Oxford, UK

Hauser I, Dehesh K, Apel K (1984) The proteolytic degradation *in vitro* of the NADPH-protochlorophyllide oxidoreductase of barley (*Hordeum vulgare* L). Arch Biochem Biophys **228:** 577–586

Hoober JK, Hughes MJ (1992) Purification and characterization of a membrane bound protease from *Chlamydomonas reinhardtii*. Plant Physiol **99:** 932–937

Kuwabara T (1992) Characterization of a prolyl endoproteinase from spinach thylakoids. FEBS Lett **300:** 127–130

Kuwabara T, Hashimoto Y (1990) Purification of a dithiothreitol sensitive tetrameric protease from spinach photosynthesis II membranes. Plant Cell Physiol **31:** 581–589

Laing WA, Christeller JT (1976) A model for the kinetics of activation and catalysis of ribulose-1,5-bisphosphate carboxylase. Biochem J **159:** 563–570

Linthorst HJM, Brederode FT, van der Does C, Bol JF (1993) Tobacco proteinase inhibitor I genes are locally but not systematically induced by stress. Plant Mol Biol **21:** 985–992

Liu XQ, Jagendorf AT (1984) ATP-dependent proteolysis in pea chloroplasts. FEBS Lett **166:** 248–252

Liu XQ, Jagendorf AT (1986) Neutral peptidases in the stroma of pea chloroplasts. Plant Physiol **81:** 603–608

Lord JM, Robinson C (1986) Role of proteolytic enzymes in the post-translational modification of proteins. *In* MJ Dalling, ed, Plant Proteolytic Enzymes, Vol 2. CRC Press, Boca Raton, FL, pp 69–80

Malek L, Bogorad L, Ayers AR, Goldberg AL (1984) Newly synthesized proteins are degraded by an ATP stimulated proteolytic process in isolated pea chloroplasts. FEBS Lett **166:** 253–257

Margossian LJ, Federman AD, Giovannoni JJ, Fischer RL (1988) Ethylene-regulated expression of a tomato fruit ripening gene encoding a proteinase inhibitor I with a glutamic residue at the reactive site. Proc Natl Acad Sci USA **85:** 8012–8016

Moore T, Keegstra K (1993) Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. Plant Mol Biol **21:** 525–537

Musgrove JE, Elderfield PD, Robinson C (1989) Endopeptidases in the stroma and thylakoids of pea chloroplasts. Plant Physiol **90:** 1616–1621

Otto S, Feierabend J (1994) Assay and comparative characterization of the proteolytic degradation of isolated small subunit and holoenzyme of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts from rye leaves. J Plant Physiol **144:** 26–33

Ozaki M, Fujinami K, Tanaka K, Amemiya Y, Sato T, Ogura N, Nakagawa H (1992) Purification and initial characterization of the proteasome from the higher plant *Spinacia oleracea.* J Biol Chem **267:** 21678–21684

Rawlings ND, Barrett A J (1994) Families of serine peptidases. Methods Enzymol **244:** 19–61

Robinson C, Ellis RJ (1984) Transport of proteins into chloroplasts: partial purification of a chloroplast protease involved in the processing of imported precursor polypeptides. Eur J Biochem **142:** 337–342

Roughan G (1994) A semi-preparative enzymic synthesis of malonyl-CoA from [^{14}C]acetate and $^{14}CO_2$: labeling in the 1, 2 or 3 position. Biochem J **300:** 355–358

Ryan CA, Walker-Simmons M (1981) Plant proteinases. *In* A Marcus, ed, The Biochemistry of Plants, Vol 6. Academic Press, New York, pp 321–350

SAS Institute (1985) SAS Users Guide. SAS Institute, Cary, NC

Schägger H, Von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range 1 to 100 kDa. Anal Biochem **166:** 368–379

Shankin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contains ClpP and ClpC functional homologues of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP dependent protease. Plant Cell **7:** 1713–1722

Sugiura M (1992) The chloroplast genome. Plant Mol Biol **19:** 149–168

Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. Annu Rev Plant Physiol Plant Mol Biol **40:** 211–233

Thayer SS, Chou HT, Raussere S, Huffaker RC (1988) Characterization and subcellular localization of aminopeptidases in senescing barley leaves. Plant Physiol **87:** 894–897

Ting IP (1968) Malic dehydrogenase in corn root tips. Arch Biochem Biophys **126:** 1–7

Van Der Valk HCPM, Van Bentum MIA, Van Loon LC (1989) Proteolytic enzymes in developing leaves of oats (*Avena sativa* L.). II Aminoacyl-2-naphthylamidases. J Plant Physiol **135:** 489–494

Vierstra RD (1993) Protein degradation in plants. Annu Rev Plant Physiol Plant Mol Biol **44:** 385–410

Waters SP, Noble ER, Dalling MJ (1982) Intracellular localization of peptide hydrolases in Wheat (*Triticum aestivum* L) leaves. Plant Physiol **69:** 575–579

Weglohner W, Subramanian AR (1992) Nucleotide sequence of a region of maize chloroplast DNA containing the 3′ end of ClpP exon 1 of rps12 and rpl20 and their cotranscription. Plant Mol Biol **18:** 415–418