Isoaspartyl dipeptidase activity of plant-type asparaginases

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Recombinant plant-type asparaginases from the cyanobacteria *Synechocystis* sp. PCC (Pasteur culture collection) 6803 and *Anabaena* sp. PCC 7120, from *Escherichia coli* and from the plant *Arabidopsis thaliana* were expressed in *E. coli* with either an N-terminal or a C-terminal His tag, and purified. Although each of the four enzymes is encoded by a single gene, their mature forms consist of two protein subunits that are generated by auto-proteolytic cleavage of the primary translation products at the Gly–Thr bond within the sequence GTI/VG. The enzymes not only deamidated asparagine but also hydrolysed a range of isoaspartyl dipeptides. As various isoaspartyl peptides are known to arise from proteolytic degradation of post-translationally altered proteins containing isoaspartyl residues, and from depolymerization of the cyanobacterial reserve polymer multi-L-arginyl-poly-L-aspartic acid (cyanophycin), plant-type asparaginases may not only function in asparagine catabolism but also in the final steps of protein and cyanophycin degradation. The properties of these enzymes are compared with those of the sequence-related glycosylasparaginases.

Key words: cyanophycin, glycosylasparaginase, isoaspartyl dipeptidase, N-terminal nucleophile hydrolase, plant-type asparaginase.

INTRODUCTION

Isoaspartyl peptides (β-aspartyl peptides) are generated by two known biological pathways. The first route is the proteolytic degradation of proteins that contain isoaspartyl residues. Such residues may be formed spontaneously under physiological conditions at aspartyl and asparaginyl residues during aging of proteins (reviewed in [1,2]). The second route is the degradation of multi-L-arginyl-poly-L-aspartic acid (cyanophycin), a reserve polymer present in most of the species of a large group of photosynthetic prokaryotes, the cyanobacteria. This non-ribosomally synthesized, branched polypeptide is depolymerized to its building block β-Asp-Arg by an enzyme called cyanophycinase [3–5].

Experiments with radiolabelled isoaspartyl dipeptides and tripeptides administered to rats indicated that a significant proportion of these peptides was metabolized rather than excreted [6]. In the same study, a peptidase activity which preferentially hydrolysed β-Asp-Gly was detected. This enzyme was partially purified from rat liver [6], but has not been characterized at a molecular level [7]. Gary and Clarke [7,8] succeeded in the purification of a β-aspartyl dipeptidase from *Escherichia coli* and characterized it in detail. The *E. coli* enzyme, which is encoded by the *iadA* gene, has no significant sequence similarity to well-characterized peptidases. However, its sequence similarity to bacterial dihydro-orotases and the structural similarity of the respective substrates, dihydro-orotate and isoaspartyl dipeptides, suggest that these hydrolases are evolutionally related and are likely to share a common mechanism [7,8]. Recently, a homologue of *iadA* has been described for *Salmonella enterica* serovar *typhimurium* [9]. Furthermore, a second enzyme that was able to hydrolyse isoaspartyl dipeptides and tripeptides was characterized in this bacterium and designated as IaaA (for isoAsp aminopeptidase) [9].

In an earlier study on the metabolism of cyanophycin, we found that crude extracts of the cyanobacterium *Synechocystis* sp. PCC (Pasteur culture collection) 6803 (*Syn.* 6803) hydrolysed β-Asp-Arg [5]. The sequenced [10] genome of *Synechocystis* contains an open reading frame (ORF), denoted *slr0179*, in CyanoBase [the database covering the genomes of *Syn.* 6803 and *Ana.* 7120 (http://www.kazusa.or.jp./cyano/cyano.html)] whose hypothetical product is distantly related (19.2% overall sequence identity) to the isoaspartyl dipeptidase IadA of *E. coli*. However, neither a knock-out of the cyanobacterial gene nor its heterologous expression in *E. coli* provided any evidence that *slr0179* encodes an isoaspartyl peptidase (M. Hejazi and W. Lockau, unpublished work). Such negative results were also obtained for another gene of *Synechocystis*, ORF *slr0807* (M. Hejazi, K. Ziegler and W. Lockau, unpublished work). Based on a mutational analysis, the hypothetical product of ORF *slr0807* had previously been suggested to function in cyanophycin degradation [11]. Its precise role remained unclear, however.

During attempts to purify the β-Asp-Arg-hydrolysing enzyme from *Synechocystis* we observed, by peptide mass 'fingerprinting', that one of the several polypeptides present in active fractions could be attributed to the asparaginase (EC 3.5.1.1) of this organism. The putative cyanobacterial asparaginase, encoded by ORF *sll0422* in the *Synechocystis* genome [10], is annotated in databases as 'plant-type asparaginase' because of its sequence similarity to the first cloned asparaginase from plants [12]. As asparaginases and β-aspartyl peptidases actually hydrolyse the same C–N bond, this observation prompted us to investigate the substrate specificity of asparaginases from two cyanobacteria. Plant-type asparaginases from *E. coli* and from

Abbreviations used: *Ana.* 7120, *Anabaena* sp. PCC 7120; α-SU, α-subunits; *A. thaliana*, *Arabidopsis thaliana* strain Landsberg erecta; CyanoBase, database covering the genomes of *Syn.* 6803 and *Ana.* 7120; GlcNAc-Asn, N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; ORF, open reading frame; PCC, Pasteur culture collection; *Syn.* 6803, *Synechocystis* sp. PCC 6803.

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Arabidopsis thaliana, respectively, were also investigated for comparison. The results demonstrate that all these plant-type asparaginases are able to hydrolyse isoaspartyl dipeptides. Furthermore, the site of post-translational proteolytic processing of the enzymes was studied. The enzymic properties of plant-type asparaginases and glycosylasparaginases are compared.

MATERIALS AND METHODS

Strains and growth conditions

Syn. 6803 and *Anabaena* sp. PCC 7120 (*Ana*. 7120) were obtained from Professor T. Börner (Humboldt-Universität zu Berlin). *E. coli* strains DH5α and BL21(DE3) were purchased from Clontech and Novagen, respectively. The cyanobacterial strains were grown in batches of 0.5 litre in culture vessels (5.5 cm diameter) or in batches of 20 litres in clear carboys at 28 °C with constant illumination (50 μmol photon $\cdot$ $m^{-2} \cdot s^{-1}$) in BG11 medium (*Synechocystis*) [13] or in a 4-fold dilution of the medium of Allen and Arnon (*Anabaena*) [14]. The cultures were vigorously bubbled with sterile air. *E. coli* strains were grown in Luria–Bertani medium [15].

Cloning and expression in *E. coli* of plant-type asparaginases from *Synechocystis*, *Anabaena*, *E. coli* and *Arabidopsis*

Standard recombinant DNA techniques [15] or minor variations thereof were used throughout. Sequence comparisons were performed using the software of Heidelberg Unix Sequence Analysis Resources (HUSAR). The genes were amplified by PCR using *Taq* DNA polymerase (New England Biolabs). Chromosomal DNA, isolated from *Synechocystis*, *Anabaena* and *E. coli* served as template. For *A. thaliana*, a cDNA library kindly provided by Professor R. Grimm (Humboldt-Universität zu Berlin) was used as template. The primer sequences, vectors and restriction enzymes used are presented in Table 1. We amplified the *Synechocystis* gene (ORF *sll0422* in CyanoBase) designated to encode a plant-type asparaginase, and genes coding for similar proteins from *Anabaena* (nt 6805–7747 of contig C158 in CyanoBase), from *E. coli* (gene *ybiK*) and from *Arabidopsis* (EMBL database accession no. Z34884). In each case, the primers contained appropriate restriction sites (Table 1) for cloning into vectors of the pET series (Novagen). The proteins were expressed with N-terminal His_{10} tags from vector pET-19b except for the *E. coli* gene *ybi*, which carried the His_6 tag of pET-22b at its C-terminus. This gene, which contains an internal *Nde*I site, was first ligated into pET-22b cut with *Nco*I and *Xho*I to yield plasmid pKM-1. Subsequently, pKM-1 was digested with *Nco*I/*Pst*I. In this way, the N-terminal signal peptide from pET-22b was removed from the insert. This insert was then ligated into plasmid pET-19b which had been digested with *Nco*I/*Pst*I so as to remove its N-terminal His_{10} tag. The resulting plasmid pKM-2 carries gene *ybiK* fused with a C-terminal His_6 tag.

Asparaginases from *Synechocystis* and *Anabaena* were expressed in *E. coli* strain BL21(DE3)pLysS (Novagen). Cultures were grown in double-strength Luria–Bertani medium (100 μg ampicillin/ml, 35 μg chloramphenicol/ml) at 37 °C on a rotatory shaker at 300 rev./min until D_{600} of approx. 0.5 was reached. Induction was initiated by addition of 1 mM isopropyl-β-D-thiogalactoside and the cultures were further incubated for 4–7 h. Recombinant asparaginases from *E. coli* and *Arabidopsis* were expressed in *E. coli* BL21(DE3) (Novagen). Cultures were grown overnight at 25–28 °C and 200 rev./min without induction.

Table 1 Primers and restriction enzymes used for cloning of genes of plant-type asparaginases in the expression vector pET-19b

For each strain, two primers are given: the primer complementary to a DNA region at the 5′-end of the respective coding sequences are given first followed by antisense primers from the 3′-end of the coding sequences in the line immediately below. Restriction sites used for cloning were introduced by the underlined nucleotides. Gene *ybiK* from *E. coli* was first cloned in vector pET-22b and subsequently transferred to pET-19b (see the Materials and Methods section). Letters N and C in the last column indicate the location of the oligohistidine tags at the N- or C-termini of the proteins.

Strain	Primer	Cloning sites	His tag
Syn. 6803	5′-ACCTAACATATGACCCCCAAAT-3′	*Nde*I	N
	5′-AGAAACCAGGGGATCCTGTTTA-3′	*Bam*HI	
Ana. 7120	5′-GCACAGGGTACATATGAAGTCACAGG-3′	*Nde*I	N
	5′-CAGTCCCCAATCTCGAGTCCCTAACTG-3′	*Xho*I	
E. coli YbiK	5′-GGACGAATACCATGGGCAAAGCAG-3′	*Nco*I	C
	5′-CGGCATCAAGTCTCGAGCTGTGTGGCAACG-3′	*Xho*I	
A. thaliana	5′-GTTATACGGCGGCATATGGTGGGGTGGGCG-3′	*Nde*I	N
	5′-ATTCCTCTCCGTCTCGAGATTTCAATTGTT-3′	*Xho*I	

Purification of recombinant asparaginases

E. coli cells from 150 ml cultures were harvested (10 min, 6500 ***g***, 4 °C), resuspended in buffer A (20 mM Tris, 0.5 M NaCl, 5 mM imidazole, adjusted to pH 8.0 with HCl) and disrupted by cavitation with ice/water cooling. Supernatants obtained after centrifugation at 14000 ***g*** (10 min, 4 °C) were loaded on to Ni-NTA columns (size 2 ml, from Qiagen). After washing with 10–20 ml of buffer A followed by 100 ml of buffer A containing 60 mM imidazole, the enzymes were eluted with 30 ml of a linear gradient of 60–1000 mM imidazole in buffer A.

Protein determination, SDS/PAGE and immunoblot analysis

Protein concentration was determined by a modified Lowry procedure [16]. SDS/PAGE was performed as in [17], with a mix of Protein Mixture 4 and 5 (Serva) as molecular mass standards. For immunoblot analysis, proteins were transferred electrophoretically to nitrocellulose membranes and immunodecorated with a polyclonal rabbit antiserum against oligohistidine (Santa Cruz Biotechnology). Antigen–antibody complexes were visualized with a peroxidase-conjugated anti-rabbit-IgG antiserum (Sigma) and developed with the ECL® Western blot detection kit (Amersham Pharmacia Biotech).

Enzyme assays

The substrates Asn, α-Asp-Arg, α-Asp-Gly, α-Asp-Leu, β-Asp-Ala, β-Asp-Gly, β-Asp-Leu, β-Asp-Lys, β-Asp-Phe, γ-Glu-Leu and N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcNAc-Asn) were purchased from Sigma and Bachem (Torrance, CA, U.S.A.), respectively. Amino acids other than Gly were in the L configuration. Asparaginase II from *E. coli* was purchased from Sigma.

Hydrolysis of asparagine and of the other substrates containing aspartate was determined by following the release of L-aspartic acid [18]. In most cases, the assay mixtures contained 67 mM $NaKPO_4$ (pH 7.4), 0.22 mM NADH (Calbiochem), 3.7 mM 2-oxoglutarate (Calbiochem), 0.36 unit malate dehydrogenase/ml (EC 1.1.1.37; Roche, Mannheim, Germany), 6 units aspartate transaminase/ml (EC 2.6.1.1; Roche), substrates at various concentrations and 0.34–11 μg purified recombinant asparaginases in a final volume of 0.8 ml and at a temperature of 35 °C. The oxidation of NADH was followed spectrophotometrically at 340 nm. At low activities, a two-step procedure was used: enzyme

preparations and substrates were incubated in 0.5 ml of 67 mM $NaKPO_4$ (pH 7.4) at 35 °C. After 30–60 min, samples were boiled, cleared by centrifugation (5 min, 17000 ***g***), and the supernatants were mixed with 0.5 ml of the assay mixture [18] lacking aspartate transaminase. The aspartate content was calculated from ΔA_{340} after addition of the transaminase. For both assay procedures, the measured activities were proportional to enzyme concentration. Glutamate release from γ-Glu-Leu was determined with glutamate dehydrogenase (EC 1.4.1.3) as described in [19].

Synthesis of β-Asp-Arg

The isodipeptide was synthesized manually on a solid support according to the Fmoc/*t*Bu chemistry. *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-tetrafluoroborate and diisopropylethyl amine were used in the activation step. As solid support Fmoc-Arg(Pmc)-TentaGel-S-PHB resin (Rapp Polymere, Tübingen, Germany) was chosen (loading: 0.19 mmol/g). The reaction was carried out in parallel using five syringes each filled with 400 mg of the resin. After Fmoc deprotection (20 % piperidine in dimethyl formamide, 2 × 5 min each), the amino acid Boc-Asp-O*t*Bu (Novabiochem, Darmstadt, Germany) was attached. For high coupling efficiency, a 4-fold molar excess of the amino acid dissolved in dimethyl formamide was activated with 1 mol *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-tetrafluoroborate/2 mol diisopropylethyl amine for 5 min and the mixture subsequently added to the resin. To improve loading, coupling was performed twice for 30 min each. The isodipeptide was deprotected and cleaved from the resin by treating with trifluoroacetic acid/phenol/water/triisobutyl silane (95:1:2:3, by vol.) for 5 h. After precipitation with cold diethylether/n-hexane (2:1), the samples were washed five times with cold diethylether/n-hexane, dissolved in 5 % acetic acid and freeze-dried. The isopeptide was analysed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF-MS) and by HPLC on a C-18 column (Vydac 201SP54). Purity of the product was > 95 %.

Mass spectrometry

The α-subunits (α-SU) of the L-asparaginases were analysed by MALDI–TOF-MS as described in [20], except that protein bands from Coomassie Brilliant Blue R250-stained SDS/PAGE were excised and digested in-gel using endoproteinase Glu-C (EC 3.4.21.19) from *Staphylococcus aureus* (Roche; 5 μg per sample) according to the manufacturer's instructions. Glu-C cleaves peptide bonds C-terminally of Glu and, with a 3000-fold lower rate, of Asp residues [21]. The resulting peptide mixtures were desalted and concentrated using $ZipTip_{C18}$ pipette tips (Millipore). The peptides were eluted from the $ZipTip_{C18}$ pipette tips with 5 μl of 50 % acetonitrile, 0.3 % trifluoroacetic acid. For the MALDI–TOF-MS analysis, samples were mixed with equal volumes of a water-saturated α-cyano-4-hydroxy cinnamic acid solution in 50 % acetonitrile and 0.3 % trifluoroacetic acid. Mass spectra were recorded in the reflectron mode of a Voyager Elite mass spectrometer (Perseptive) with delayed extraction. Instrument parameters were 20 kV accelerating voltage, 70 % grid voltage, 0.050 % guide-wire voltage, 100 ns delay, low-mass gate of 500 and summation of 256 laser shots. A mass accuracy of approx. 30 p.p.m. was obtained by internal calibration using three synthetic peptides. Proteins were identified by searches in SWISS-PROT. Searches were performed using the program MS-FIT (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm), reducing the proteins of the database to the proteins of the respective organism and allowing for a mass tolerance of 0.1 Da. Partial enzymic cleavages leaving two cleavage sites, acetylation of N-termini of proteins (Acet-N), pyroglutamate formation at N-terminal Glu of peptides (pyro-Glu), oxidation of Met (Met-ox) and modification of Cys by acrylamide (Cys-am) were considered in the database searches. For asparaginase of *Anabaena*, which is not yet included in protein databases, the program MS-DIGEST (http://prospector.ucsf.edu/ucsfhtml3.4/msdigest.htm) was used to perform a computer simulation of the enzymic digestion on the basis of the amino-acid sequence deduced from the gene sequence reported in CyanoBase.

RESULTS

Expression of four plant-type asparaginases in *E. coli* and analysis of their subunit structure

Our initial goal was to characterize an isopeptidase of the cyanobacterium *Syn.* 6803 that would hydrolyse β-Asp-Arg, the building block and immediate degradation product [5] of the reserve polymer cyanophycin (multi-L-arginyl-poly-L-aspartic acid). The observation, by peptide mass mapping, that fractions enriched in such an activity contained the product of gene (ORF) *sll0422* encoding a putative plant-type asparaginase suggested that these asparaginases may hydrolyse isoaspartyl dipeptides. A BLASTP search [22] revealed approx. 20 genes annotated to encode plant-type asparaginases (EC 3.5.1.1) as well as some more distantly related genes annotated to encode glycosylasparaginases (EC 3.5.1.26). We decided to express His-tagged versions of four putative asparaginases of the plant type in *E. coli* and to study their response towards asparagine, various aspartyl dipeptides and towards GlcNac-Asn, a substrate of glycosylasparaginases. The deduced primary sequences of these enzymes are aligned in Figure 1. The encoded proteins are of similar length (315–329 amino acid residues). In pairwise comparisons using the ClustalW program [23], the overall sequence identities (similarities) ranged from 28.8 % (38.0 %) for *Synechocystis* versus *Arabidopsis* to 65.2 % (73.9 %) for *Anabaena* versus *Synechocystis*.

The cloning procedures, described in the Materials and Methods section, resulted in recombinant proteins (primary translation products) that carried a His_6 tag at the C-terminus (asparaginase from *E. coli*) or a His_{10} tag at the N-terminus (asparaginases from *Synechocystis*, *Anabaena* and *Arabidopsis*). The proteins were purified from extracts of *E. coli* by chromatography on Ni-NTA columns. The purified proteins were analysed by SDS/PAGE (Figure 2A). In all cases, two strongly stained polypeptide bands in the region corresponding to apparent molecular masses of 10–25 kDa were observed. The molecular masses of the rather faint bands above 30 kDa were close to the masses calculated for the respective primary translation products. These polypeptides reacted with an antiserum against the His tag (Figure 2B), irrespective of its location at the N-terminus (*Arabidopsis*, *Anabaena* and *Synechocystis*) or C-terminus (*E. coli*). Thus, these polypeptides appear to represent unprocessed primary translation products. Even in the purified fractions, the abundance of these polypeptides decreased with time (not shown). The polypeptides with higher electrophoretic mobilities are obviously products of proteolytic processing of the precursors. Their reaction with the His-tag antiserum (Figure 2B) provided evidence that the polypeptides designated as α-SU were derived from N-terminal parts of the respective precursors. This conclusion was corroborated by peptide mass mapping (see the Materials and Methods section) of the α-SU from *Arabidopsis*, *E. coli* and *Synechocystis*. With sequence coverages ranging over 31–63 %, none of the observed peptide masses matched peptides outside the proposed sequences of the α-SU (results not shown).

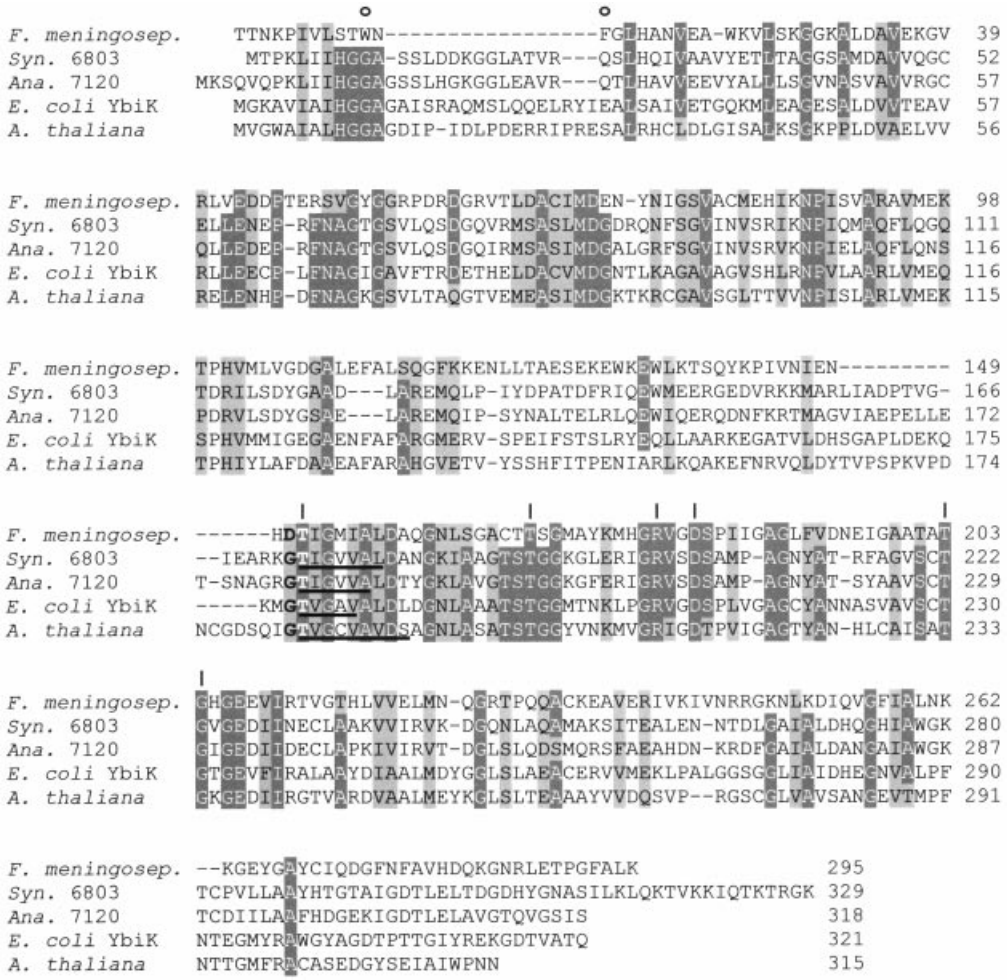

Figure 1 Alignment of the deduced amino-acid sequences of the precursors of glycosylasparaginase from *Flavobacterium meningosepticum* and of four plant-type asparaginases from various organisms

The ClustalW program [23] was used (www2.ebi.ac.uk/clustalw). The sequences are deduced from: *Ana.* 7120, nucleotides 6805–7747 of contig C158 in CyanoBase; *A. thaliana* strain Landsberg erecta in EMBL database Z34884; *E. coli*, gene *ybiK*; *F. meningosep., Flavobacterium meningosepticum* in GenBank® Nucleotide Sequence Database with accession no. U08028; *Syn.* 6803, ORF *sll0422* in CyanoBase. The sequence of the *Flavobacterium* glycosylasparaginase is shown without its signal sequence. Residues identical in at least four sequences of the alignment are shown in white on a dark-grey background and conserved and semi-conserved substitutions are shown in black on a light-grey background. Maturation of the precursors involves hydrolysis between the amino acids given in bold letters (DT and GT, respectively). Amino-acid residues identified by peptide sequencing of the β-SU of mature enzymes are underlined. Identification of the Cys residue in the *A. thaliana* β-SU was somewhat uncertain. A small vertical line above the sequences identifies conserved residues located in the active site of *F. meningosepticum* and human glycosylasparaginases that are also present in plant-type asparaginases; ○, residues that contribute to binding of the *N*-acetylglucosamine moiety of the natural substrate of glycosylasparaginases (see the Discussion section).

N-terminal peptide sequences (underlined in Figure 1) were determined for the polypeptides designated as β-SU (β-subunits) by automated Edman degradation. In all the four cases, the N-terminal amino acid generated by the proteolytic processing was the threonine residue within the consensus sequence -GTV/IG-. We also attempted to obtain information about the C-terminus of the α-SU by peptide mass mapping. No such information was obtained when the four α-SU were digested with trypsin. Probably, the high abundance of Arg and Lys residues in the primary sequences preceding the predicted cleavage site (cf. Figure 1) resulted in peptides from the respective C-termini that were too small to be analysed by the MALDI–TOF-MS method used. However, inspection of these regions of the amino-acid sequences predicted that the C-terminus of the α-SU of the enzyme protein from *Arabidopsis* should be covered by analysis of peptides obtained by in-gel digestion with endoproteinase Glu-C. In the mass spectrum (Figure 3), 11 of the measured peptide masses could be assigned to the α-SU (amino-acid residues 1–182), resulting in a sequence coverage of 57%. Two of the detected peptide masses indicate that the asparaginase precursor of *Arabidopsis* is cleaved C-terminally after Gly-182: the peptide with an m/z ratio of 1949.9 can be assigned to amino-acid residues 165–182 and the peptide with an m/z ratio of 2820.29 to residues 158–182, respectively. Both fragments can only occur if the asparaginase precursor is cleaved at the position indicated in Figure 1. It is evident that the asparaginase precursor is proteolytically processed by hydrolysis of a single peptide bond rather than by removal of an internal peptide. The four asparaginases studied here eluted from a calibrated Superdex 200 column (Pharmacia) at a volume corresponding to approx. 70 kDa (results not shown), suggesting that the mature enzymes have the subunit structure $\alpha_2\beta_2$.

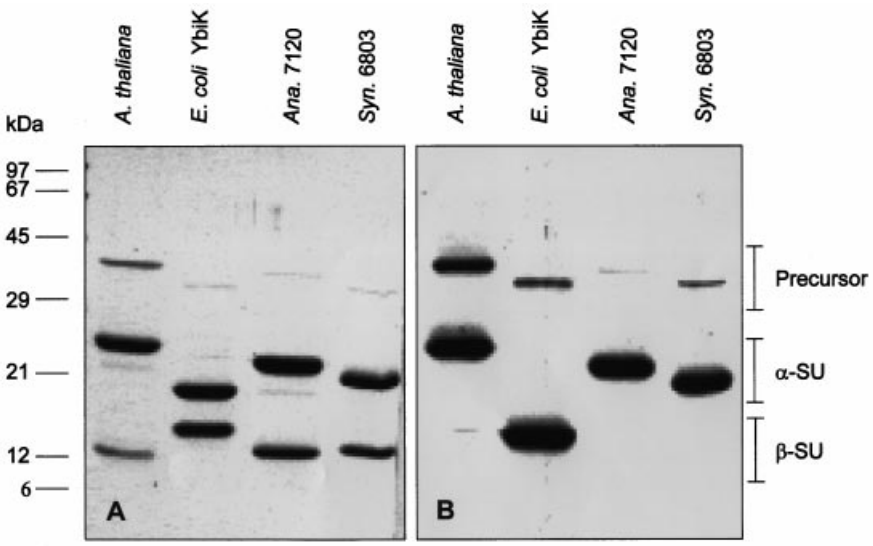

Figure 2 SDS/PAGE and immunoblot analysis of purified samples of the plant-type asparaginases studied

Proteins were expressed in *E. coli* either with a C-terminal (*E. coli* enzyme) or N-terminal His-tag (enzymes from *A. thaliana*, *Anabaena* and *Synechocystis*) and purified by chromatography on Ni-NTA columns. (**A**) SDS/PAGE stained with Coomassie Blue R-250. (**B**) Western blot immunodecorated with an antiserum against the His-tags. Locations of the primary translation products (precursor) and of the two subunits of the mature enzymes (α-SU and β-SU, respectively, corresponding to the N- and C-terminal parts of the precursors) are shown on the right-hand side. See text for further details.

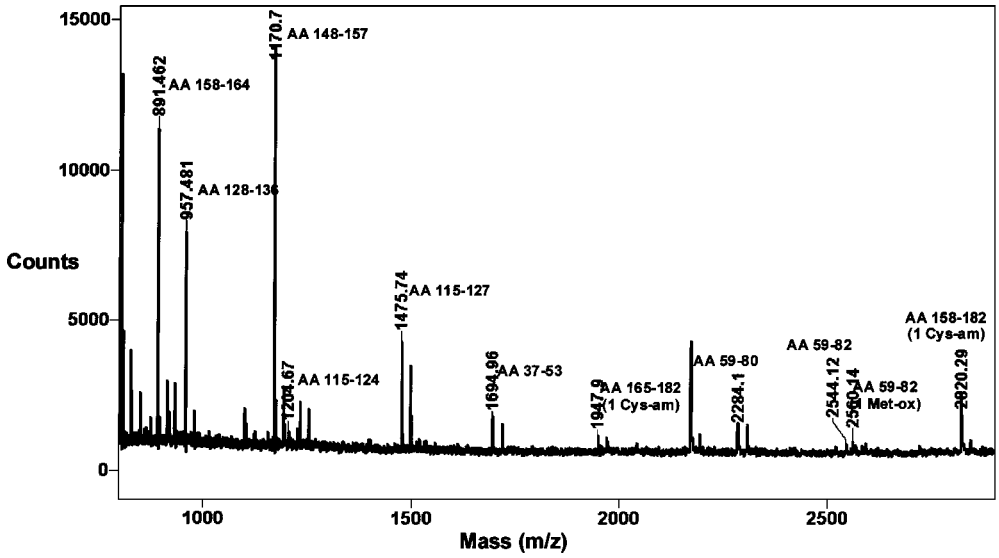

Figure 3 MALDI-MS spectrum of the α-SU (residues 1–182) of the L-asparaginase of *A. thaliana* digested with endoproteinase Glu-C

Masses (*m/z*) are given for the peaks that could be assigned to peptides within this subunit, yielding a sequence coverage of 57%. None of the experimental peptide masses matched a peptide outside the residues 1–182. The observed peptides with *m/z* ratios of 1949.9 and 2820.29 represent the masses expected for amino-acid residues 165–182 and 158–182, respectively, of the *Arabidopsis* asparaginase. Both fragments can only occur if the enzyme is cleaved during maturation at the position indicated in Figure 1. Cys-am, modification of cysteine by acrylamide; Met-ox, oxidation of methionine.

Asparaginase activity

Consistent with their annotation as asparaginases, all investigated enzymes hydrolysed L-asparagine (Table 2). The apparent K_m values for this substrate varied considerably, ranging from 0.6 mM (*Synechocystis*) to > 4.0 mM for the *E. coli* and *Arabidopsis* enzymes. The reported K_m values of plant-type asparaginases purified from plants range over 3–12 mM, approx. 100-fold higher than those of most animal and bacterial enzymes [24]. The specific activity of the *Arabidopsis* asparaginase with asparagine as well as with isoaspartyl dipeptides as substrates was much lower than that of the bacterial enzymes (Table 2). From the data reported by Chang and Farnden [24], one can calculate a specific activity of the asparaginase purified from *Lupinus arboreus* (lupin) seeds of $\approx 0.7\ \mu mol \cdot min^{-1} \cdot mg^{-1}$ protein at a

Table 2 Hydrolysis of asparagine and various β-aspartyl dipeptides by recombinant plant-type asparaginases and by asparaginase II (AnsB) of *E. coli*

The reported apparent Michaelis constants and the specific V_{max} values were reproducible within ±20%. Low affinity of the asparaginase from *A. thaliana* to the substrates precluded determination of K_m and V_{max} values by the assay used. Details of cloning, expression and assay are described in the Materials and Methods section. Asparaginase II of *E. coli*, which is not a plant-type asparaginase, was purchased from Sigma. Amino acids were in the L configuration. n.d., activity not detected.

Enzyme from	Substrate	Rate* at [S] = 2 mM	Specific V_{max}*	Apparent K_m (mM)	V_{max}/K_m
Syn. 6803	Asn	1.2	1.6	0.66	2.4
	β-Asp-Ala	4.8	7.3	1.0	7.3
	β-Asp-Arg	18.0	21.0	0.32	66
	β-Asp-Gly	1.3	2.4	1.6	1.5
	β-Asp-Leu	12.5	14.7	0.33	45
	β-Asp-Lys	23.0	29.4	0.55	53
	β-Asp-Phe	18.5	23.6	0.56	42
Ana. 7120	Asn	1.1	2.2	2.0	1.1
	β-Asp-Ala	6.95	12.3	1.54	8.0
	β-Asp-Arg	5.0	5.7	0.27	21
	β-Asp-Gly	2.1	2.7	0.52	5.2
	β-Asp-Leu	4.0	4.5	0.26	17
	β-Asp-Lys	17.6	22.2	0.53	42
	β-Asp-Phe	15.0	20.1	0.68	30
E. coli YbiK	Asn	8.2	–	> 4.0	–
	β-Asp-Ala	59.3	66.8	0.25	267
	β-Asp-Arg	11.5	16.2	0.82	20
	β-Asp-Gly	n.d.	–	–	–
	β-Asp-Leu	143.0	180.0	0.52	346
	β-Asp-Lys	37.0	60.0	1.24	48
	β-Asp-Phe	48.0	50.5	0.12	421
A. thaliana	Asn	0.40	–	> 4.0	–
	β-Asp-Ala	0.32	–	> 4.0	–
	β-Asp-Arg	n.d.	–	–	–
	β-Asp-Gly	0.14	–	> 4.0	–
	β-Asp-Leu	0.11	–	> 4.0	–
	β-Asp-Lys	n.d.	–	–	–
	β-Asp-Phe	0.48	–	> 4.0	–
E. coli AsnB	Asn	50.0	324.5	0.12	2704
	β-Asp-Ala	n.d.	–	–	–
	β-Asp-Arg	n.d.	–	–	–
	β-Asp-Gly	n.d.	–	–	–
	β-Asp-Leu	n.d.	–	–	–
	β-Asp-Lys	n.d.	–	–	–
	β-Asp-Phe	n.d.	–	–	–

* Rates are given as μmol aspartate released $min^{-1} \cdot mg^{-1}$ protein.

concentration of 2 mM asparagine. This value is similar to the one determined for the recombinant *Arabidopsis* enzyme (Table 2). A low specific activity may thus be an intrinsic property of the enzymes from plants. None of the four plant-type asparaginases studied here hydrolysed glutamine (results not shown).

Hydrolysis of dipeptides and of GlcNAc-Asn

None of the plant-type asparaginases hydrolysed the α-aspartyl dipeptides tested (α-Asp-Arg, α-Asp-Gly, α-Asp-Leu) at a measurable rate (results not shown). However, various β-aspartyl dipeptides served as substrates (Table 2). The affinity of the *Arabidopsis* enzyme towards all β-aspartyl dipeptides was low, the rate of hydrolysis being linearly dependent on the substrate concentration up to 4 mM, the highest concentration tested. It is worth noting that the two cyanobacterial asparaginases had a rather high affinity for the isoaspartyl dipeptides containing a basic amino acid, i.e. β-Asp-Arg (apparent $K_m \approx 0.3$ mM) and β-Asp-Lys ($K_m \approx 0.5$ mM). The corresponding values for the *E. coli* enzyme were approx. 2.5-fold higher, whereas hydrolysis of these isodipeptides by the *Arabidopsis* enzyme was not detected even at concentrations of 4–5 mM. None of the enzymes hydrolysed GlcNAc-Asn, the principal substrate of glycosylasparaginases. The enzymes did not cleave γ-Glu-Leu. Together with their inability to deamidate glutamine (see above), this suggests a specificity for aspartate at the N-terminal position.

The V_{max}/K_m values listed in Table 2 show that the asparaginases from the two cyanobacteria are rather unspecific for the amino acid bound to the β-carboxyl group of aspartate. Only amino acids with a small side chain (glycine and alanine) strongly reduced the catalytic efficiency, resulting in V_{max}/K_m values in the range of those determined for asparagine. The enzyme from *E. coli*, however, is more specific for the β-bound residue of isodipeptides (Table 2). It should be noted that *E. coli* is known to express a second enzyme with β-aspartyl dipeptidase activity [8] that may complement its plant-type asparaginase in some physiological functions.

Asparaginase II of *E. coli* does not hydrolyse β-aspartyl dipeptides

Much better investigated than the *ybiK* gene product are two asparaginases of *E. coli* that are encoded by the genes *ansA* and *ansB*, respectively (reviewed in [25,26]). AnsA and AnsB show significant sequence similarity. For comparison, we have characterized the specificity of one of these enzymes, the so-called asparaginase II (AnsB). Asparaginase II readily hydrolysed asparagine, with a high V_{max} and a low K_m value (Table 2). However, none of the isoaspartyl dipeptide substrates was cleaved by this enzyme at a measurable rate (Table 2). It is evident that isoaspartyl dipeptidase activity is not a general feature of asparaginases.

DISCUSSION

Plant-type asparaginases as well as the sequence-related glycosylasparaginases are the so-called N-terminal nucleophile hydrolases, a superfamily of enzymes that was first recognized by Brannigan et al. [27]. Amino-acid sequences of members of this superfamily may vary considerably but their structures often appear to be similar [28]. N-terminal nucleophile hydrolases usually mature via proteolytic processing of single-chain precursors. The newly generated N-terminal amino acid, either a threonine, serine or cysteine, participates both as a base (α-amino group) and also as a nucleophile (side chain hydroxy or thiol group) in amide-bond hydrolysis. Like glycosylasparaginases [29], the precursors of plant-type asparaginases are processed by hydrolysis of a single peptide bond (Figures 2 and 3), as already suggested in a previous study [12] on an asparaginase from lupin seeds. The asparaginase precursors are cleaved between Gly-172 and Thr-173 (numbering according to the *Synechocystis* sequence, Figure 1) within the consensus sequence -GTI/VGV/A-. The intensely studied glycosylasparaginases from *Homo sapiens* and *Flavobacterium meningosepticum* are processed by hydrolysis between Asp and Thr within the consensus -DTIGM- [30,31]. The cleavage sites are located at similar positions in the primary sequences of plant-type asparaginases and glycosylasparaginases (Figure 1). In the case of the *Flavobacterium* glycosylasparaginase, the Asp residue preceding the nucleophile Thr has been shown to be 'virtually required' [32] for autoproteolytic processing. As this residue is not conserved in the asparaginase sequences (Figure 1), the structure of the

autoproteolytic centre of the asparaginases should differ considerably from that reported for glycosylasparaginase [33]. The enzyme properties of most of the products of the more than 30 genes in the databases with significant sequence similarity to glycosylasparaginases and plant-type asparaginases have not been determined experimentally. Thus, it is not possible at present to decide whether the differences in the two consensus sequences given above are distinctive in general, for plant-type asparaginases and glycosylasparaginases, respectively.

The N-terminal nucleophile Thr of the asparaginases as well as the glycosylasparaginases is followed by a highly conserved stretch of ≈ 55 amino acids. Five of the residues of this stretch, marked in Figure 1 by vertical lines above the sequences, are known from structural analyses to be located in the active centre of glycosylasparaginases and are known to interact with the reaction product aspartate [30,31]. Probably, these residues have a similar role in plant-type asparaginases.

Enzyme properties of plant-type asparaginases have not been reported before except for the recent work on IaaA of *Salmonella enterica* [9]. IaaA is a homologue of asparaginase YbiK of *E. coli*. The substrate spectrum of the *Salmonella* enzyme is similar to that of YbiK, including its inability to hydrolyse GlcNAc-Asn. As judged from the substrate specificities of these five enzymes (Table 2 and [9]), which are from cyanobacteria, bacteria and a higher plant, the isoaspartyl peptidase activity is a widespread and probably even a general property of plant-type asparaginases, although the specificities of the individual enzymes are not completely identical (Table 2). On the contrary, asparaginase II of *E. coli*, a protein with a primary structure clearly different from plant-type asparaginases, did not accept isoaspartyl dipeptides (see the Results section). A special feature of the cyanobacterial asparaginases is their high catalytic efficiency towards β-Asp-Arg (Table 2). Thus, the cyanobacterial enzymes may be responsible for the last step of degradation of the reserve polymer cyanophycin (see the Introduction section).

The available data (Table 2 and [9]) indicate that asparagine is not a kinetically preferred substrate of plant-type asparaginases. For this reason, the enzyme from *Salmonella* was named IaaA (for isoAsp aminopeptidase) by Larsen et al. [9]. This annotation may be acceptable for heterotrophic bacteria that contain further enzymes capable of hydrolysis of asparagine with high affinity, like asparaginases AnsA and AnsB of *E. coli* [25,26]. However, based on the analysis of its genome [10], the photoautotrophic cyanobacterium *Synechocystis* contains just one gene encoding an asparaginase, and this is the ORF *sll0422* whose product was studied here. Higher plants contain several enzyme species able to deamidate asparagine [12,24,34]. Asparaginases that have been isolated from developing plant seeds are of the 'plant type', with a low affinity towards asparagine (K_m values ranging over 3–12 mM). Concentrations of soluble asparagine in plants, however, can be quite high, e.g. as high as 21 mM in seeds of *L. arboreus* [24]. Thus, in plants even the enzymes with a low affinity could be used efficiently as asparaginases. We therefore suggest that the name 'plant-type asparaginases' be retained at least in these cases.

What distinguishes plant-type asparaginases from glycosylasparaginases? The well-established physiological role of glycosylasparaginases is the cleavage of the protein-to-carbohydrate linkage (GlcNAc-Asn) during degradation of N-glycosylated proteins. Although GlcNAc-Asn is their preferred substrate, some but not all glycosylasparaginases are able to deamidate asparagine [35], and the human enzyme has been shown to hydrolyse β-aspartyl peptides [36]. Thus, the only clear-cut difference in the enzyme properties of glycosylasparaginases and plant-type asparaginases is that the former enzymes hydrolyse GlcNAc-Asn, whereas the latter ones do not (Table 2 and [9]). Two conserved aromatic residues located in the vicinity of the catalytic Thr in mature glycosylasparaginases, Trp-11 and Phe-13, contribute to the binding of the carbohydrate (*N*-acetylglucosamine) moiety of their substrate GlcNAc-Asn [30,31]. No counterparts of these residues are found in the N-terminal regions of the primary structures of plant-type asparaginases (Figure 1). Occurrence of these two aromatic residues in glycosylasparaginases may thus help in distinguishing them from plant-type asparaginases.

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