Heat-stable ureases from two filamentous cyanobacteria

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Ureases of the cyanobacteria *Leptolyngbya boryana* (*Plectonema boryanum*) PCC 73110 and *Anabaena/Nostoc* PCC 7120 were purified more than 1500-fold to homogeneity by heat treatment and liquid chromatography, reaching specific activities of up to 350 U (mg protein)⁻¹. Both enzymes had a molecular mass of 220 kDa, as shown by native PAGE, and consisted of three subunits (α , β , γ) with molecular masses of 66 kDa (α), 18 kDa (*Leptolyngbya*; β) or 14 kDa (*Anabaena/Nostoc*; β) and 11 kDa (γ). The enzyme of *Leptolyngbya* exhibited maximum activity at pH 8.2 and 60 °C, and that of *Anabaena/Nostoc* at pH 8.5 and 65 °C; the K_m was 0.25 mM urea for both organisms. Almost identical specific activities were observed in cells grown with urea, ammonia, nitrate or dinitrogen as the source of nitrogen. The ureases from both organisms were heat-stable; no loss of activity was observed during incubation at 70 °C for 15 min.

Keywords: urease, purification, cyanobacteria, *Leptolyngbya*, *Anabaena/Nostoc*

INTRODUCTION

The ability to metabolize urea is widespread among micro-organisms (Barth & Michel, 1971); in some fungi and green algae, urea is first carboxylated to yield allophanate, which is then hydrolysed to two molecules of ammonia and carbon dioxide (Leftley & Syrett, 1973; Roon & Levenberg, 1972). Cyanobacteria are well known to utilize urea as a source of nitrogen for growth (McLachlan & Gorham, 1962; Berns *et al.*, 1966). In cyanobacteria, as in all other urea-utilizing bacteria studied so far (for a review, see Mobley & Hausinger, 1989, and references therein), the enzyme urease (urea amidohydrolase; EC 3.5.1.5) catalyses the hydrolysis of urea to ammonia and carbamate; carbamate decomposes spontaneously to carbon dioxide and a second molecule of ammonia.

Earlier studies concerning the structural properties of bacterial ureases led to conflicting results. Most ureases, e.g. those from *Klebsiella aerogenes* (*K. pneumoniae*) (Mulrooney & Hausinger, 1990), *Proteus mirabilis* (Breitenbach & Hausinger, 1988), *Proteus vulgaris* (Mörsdorf & Kaltwasser, 1990) or *Providencia stuartii* (Mulrooney *et al.*, 1988), were shown to be composed of three different types of subunit, designated α , β and γ . In contrast, the ureases of *Bacillus pasteurii* and *Selenomonas ruminantium* were first described as being homopolymeric, consisting only of the large subunits (Christians & Kaltwasser, 1986; Hausinger, 1986). Subsequent studies

on the enzyme of *S. ruminantium* (Todd & Hausinger, 1987) and *B. pasteurii* (T. Jahns, unpublished), however, revealed that ureases from these Gram-positive organisms are similar with regard to their subunit composition (α , β , γ) to those of the Gram-negative bacteria studied. It appears that the small subunits may have been overlooked in SDS-PAGE gels which were too low in acrylamide concentration.

The ureases of the cyanobacteria *Spirulina maxima* (Carvajal *et al.*, 1982), *Anabaena doliolum* (Rai, 1989) and *Anabaena cylindrica* (Argall *et al.*, 1992) appear to differ from those of other bacteria, being homopolymeric enzymes, composed of six subunits of molecular mass between 32 and 38 kDa. Apparently, however, none of these cyanobacterial enzymes were purified to homogeneity. We therefore started a more detailed study of the ureases of the two filamentous cyanobacteria, *Leptolyngbya boryana* (*Plectonema boryanum*) PCC 73110 and *Anabaena/Nostoc* PCC 7120. The ureases from these organisms turned out to be thermostable enzymes similar to other ureases of prokaryotic origin with regard to their subunit composition, but different from the cyanobacterial ureases described so far.

METHODS

Materials. All chemicals and reagents were of at least analytical grade. Molecular mass standard proteins were from Boehringer or Sigma. Q-Sepharose FF, Phenyl-Sepharose HL, Sephacryl

S300HR and Mono-Q were purchased from Pharmacia. The media and the buffers used for enzyme purification were prepared with Milli-Q-purified water (Millipore).

Organisms, growth conditions and preparation of extracts.

Leptolyngbya boryana (*Plectonema boryanum*) PCC 73110 and *Anabaena/Nostoc* PCC 7120 were obtained from the Pasteur Culture Collection, Paris, France. The cells were grown to late exponential phase with slow shaking or stirring at 28 °C and 3000 lx (Osram 36W/21, Lumilux cool white) in medium BG11 (Rippka *et al.*, 1979), to which 10 mM filter-sterilized NaHCO₃ was added. In one set of experiments, sodium nitrate was replaced by other nitrogenous compounds; 15 mM sodium chloride was added to these media. Purity was assessed by microscopic examination and by regular streaking of the strains on solidified BG11, supplemented with 0.2% (w/v) glucose and 0.02% (w/v) Casamino acids (Rippka *et al.*, 1979). Cells were harvested by centrifugation (20000 g for 30 min at 4 °C) and washed twice in 50 mM sodium phosphate, 1 mM Na₂EDTA, pH 7.0 (buffer A). Cells were disintegrated by ultrasonification of cell suspensions containing approximately 0.3 g wet weight per ml buffer A. Ultrasonic treatment lasted a total of 30 s ml⁻¹, applied in periods of 15 s with intermittent cooling in an ice bath, using a Branson sonifier B12 at no more than 60 W, and was followed by centrifugation for 30 min at 45000 g in order to obtain cell-free extracts.

Measurement of enzyme activity and protein. Urease activity was determined by measuring the amount of ammonia liberated from urea by the indophenol method (Chaney & Marbach, 1962) or in an NADH-dependent coupled enzyme assay according to the method of Kaltwasser & Schlegel (1966). Fractions eluting from the Phenyl-Sepharose column and containing high amounts of ammonium were only qualitatively assayed for urease activity by a pH indicator assay using phenol red (Hamilton-Miller & Gargan, 1979). One unit of enzyme activity (U) is defined as the decomposition of 1 µmol urea min⁻¹ at 30 °C and pH 8.0; specific activities are expressed as U (mg protein)⁻¹. Protein was determined according to the Lowry method using bovine serum albumin as a standard.

Electrophoresis. For the determination of the molecular mass of the subunits, samples from different stages of purification were analysed by SDS-PAGE in a Biometra Minigel system according to Laemmli (1970). Native gel electrophoresis was carried out at 4 °C in horizontal ultrathin gels (240 × 125 × 0.5 mm) at a gradient of 4 to 32% polyacrylamide (C = 3%). The gels were separated at 8000 V h and subsequently silver-stained for protein visualization according to Merril *et al.* (1981) or for activity as described by Fishbein (1969).

Purification of urease. All the steps in the purification of the enzyme from both cyanobacteria were carried out at between 22 and 28 °C, except size-exclusion chromatography, which was performed at 4 °C, and heat treatment, performed at 70 °C.

(i) **Heat treatment.** Extracts of cells grown in medium BG11 were heated with continuous stirring at 70 °C for 15 min; they were centrifuged for 30 min at 45000 g and 4 °C and the pellets were discarded.

(ii) **Binding to Q-Sepharose.** The supernatant was loaded onto a Q-Sepharose FF column (2 cm² × 10 cm) connected to an HPLC system (LKB, Bromma; pump 2150, detector 2158 uvicord SD, fraction collector 2211 superrac, controller 2152 LC) and equilibrated with buffer A. Proteins were eluted using a linear gradient from 0.1 to 1.0 M NaCl in buffer A with a flow rate of 2.0 ml min⁻¹. Elution of protein was followed at 280 nm, and five 4.0 ml fractions with the highest urease activity, eluting at a concentration of about 0.43 M NaCl (*Leptolyngbya*) or 0.40 M NaCl (*Anabaena/Nostoc*), were pooled.

(iii) **Hydrophobic interaction chromatography.** The fractions containing urease activity (total volume 20 ml) were adjusted to 1.0 M (NH₄)₂SO₄ by adding 10 ml 3.0 M (NH₄)₂SO₄ and loaded onto a Phenyl-Sepharose HL column (2 cm² × 12 cm) connected to the LKB HPLC system, previously equilibrated with buffer A, containing 1 M (NH₄)₂SO₄. In a linear gradient from 1.0 to 0 M (NH₄)₂SO₄, the enzyme of both strains was eluted with a flow rate of 2.0 ml min⁻¹ at 0.40 M (NH₄)₂SO₄.

(iv) **Size-exclusion chromatography.** The two most active 4 ml fractions from the Phenyl-Sepharose column were pooled and applied to a Sephacryl S300HR column (4.9 cm² × 99 cm) connected to the LKB HPLC system. The elution buffer used was buffer A containing 0.1 M NaCl, with a flow of 0.45 ml min⁻¹. Fractions of 4.5 ml were collected and assayed for urease activity.

(v) **Anion-exchange chromatography.** The three most active fractions from size-exclusion chromatography were pooled, loaded onto a Mono-Q HR5/5 column connected to the LKB HPLC system and eluted using a linear gradient from 0.1 to 1.0 M NaCl in buffer A with a flow rate of 1 ml min⁻¹; urease was eluted at approximately 0.42 M NaCl (*Leptolyngbya*) or 0.40 M NaCl (*Anabaena/Nostoc*).

Reproducibility of results. The table and the figure show representative results from single experiments, which were repeated at least twice. Determination of the K_m values was repeated four times and K_m was calculated from Lineweaver-Burk plots. Linear correlations were fitted by regression analysis, yielding values for r of between 0.988 and 0.996. Other results are mean values from at least two independent experiments. SD values did not exceed ± 20% of the mean value and are not indicated.

RESULTS AND DISCUSSION

Purification of cyanobacterial urease

Leptolyngbya boryana PCC 73110 and *Anabaena/Nostoc* PCC 7120 showed consistently low levels of urease of about 0.12 U (mg protein)⁻¹ and 0.08 U (mg protein)⁻¹, respectively. Almost identical specific activities were observed after the growth of both strains in media containing nitrate (5 mM), ammonium (5 mM) or urea (2.5 mM). These findings are similar to observations with *Anabaena cylindrica* (Mackerras & Smith, 1986) and *Anabaena variabilis* (Ge *et al.*, 1990), where urease synthesis was shown to occur regardless of the nitrogen source used for growth; in *Anabaena doliolum* and *Anacystis nidulans*, however, urease synthesis was repressed by ammonium (Singh, 1990, 1991).

After purification of urease from nitrate-grown cells of *L. boryana* PCC 73110 by heat treatment and ion-exchange chromatography on Q-Sepharose and Mono-Q, hydrophobic interaction chromatography on Phenyl-Sepharose, and size-exclusion chromatography on Sephacryl S300HR, an enrichment of more than 1500-fold was obtained (Table 1); similar results were obtained for the enzyme of *Anabaena/Nostoc* PCC 7120 (data not shown). In order to obtain a homogeneous enzyme preparation, only fractions exhibiting the highest urease activities were used for the subsequent enrichment by column chromatography; this procedure led to the comparatively low recovery of 8%. The highest specific activities obtained

Table 1. Purification of urease from *Leptolyngbya boryana* PCC 73110

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]*	Recovery (%)	Purification factor
Crude extract	3574	460	0.129	100	1
Heat treatment	294	384	1.306	84	10
Q-Sepharose	7	286	40.86	62	315
Phenyl-Sepharose	ND	ND	ND	ND	ND
Sephacryl S300HR	ND	72	ND	16	ND
Mono-Q HR5/5	0.184	36.8	202.3	8	1568

ND, Not determined.

* Specific activities were determined by measuring the amount of ammonia liberated from urea, except in the fractions eluted from Phenyl-Sepharose, where urease was determined qualitatively by a pH indicator assay using phenol red, as described in Methods.

Fig. 1. Native pore gradient electrophoresis (a) or SDS-PAGE (b) of urease from *Leptolyngbya boryana* PCC 73110 and *Anabaena/Nostoc* PCC 7120. (a) A homogeneous urease sample with a specific activity of approximately 200 U (mg protein)⁻¹ was separated by non-denaturing electrophoresis in 4–32% polyacrylamide gradient gels. Lanes 1–3, silver stain: lane 1, molecular mass standards [ferritin (450 kDa; F), catalase (240 kDa; C), aldolase (158 kDa; A), bovine serum albumin (67 kDa; B) and egg albumin (43 kDa; E)]; lane 2, urease from *Anabaena/Nostoc*; lane 3, urease from *L. boryana*. Lanes 4 and 5, activity stain: lane 4, urease from *Anabaena/Nostoc*; lane 5, urease from *L. boryana*. (b) Urease (0.6 µg per lane: lane 1, *L. boryana*; lane 2, *Anabaena/Nostoc*) was subjected to SDS-PAGE in a 15% gel. Proteins were visualized by silver staining. Lane 3, protein standards.

were between 200 and 350 U (mg protein)⁻¹ for the urease of both strains, measured at 30 °C. This activity is more than 20 times higher than that observed in purified preparations of other cyanobacterial ureases (Carvajal *et al.*, 1982; Rai, 1989; Argall *et al.*, 1992); in other bacteria, however, much higher specific activities of up to 180 000 U (mg protein)⁻¹ have been observed (Mobley & Hausinger, 1989).

Kinetic properties and heat stability

Using the purified enzyme for all subsequent studies, a pH optimum of 8.2 (*L. boryana*) or 8.5 (*Anabaena/Nostoc*) in both buffer A and 50 mM disodium diethylbarbiturate

was determined. The urease of both cyanobacteria exhibited Michaelis–Menten kinetics with a K_m value of 0.25 mM urea, measured at optimal pH and 30 °C. Maximum activity was obtained at 60 °C (*L. boryana*) or 65 °C (*Anabaena/Nostoc*).

The enzyme of both strains was remarkably heat-stable; no loss of activity was observed upon incubation for 15 min at temperatures between 30 and 70 °C. At temperatures above 72 °C, a rapid irreversible inactivation occurred. Similar heat-stability was described for the urease of *Anabaena variabilis* (Ge *et al.*, 1990), and the urease of *Providencia rettgeri* was stable even at temperatures of up to 80 °C, but only in the presence of its substrate; the activity

was rapidly lost in its absence (Magana-Plaza *et al.*, 1971). No such protective effect of urea was detected with the urease from *L. boryana* or *Nostoc/Anabaena* (results not shown). Both purified enzymes lost approximately 40 % of activity within 5 d upon storage at 4 °C; immediate and complete loss of activity was observed upon incubation at -20 °C in buffer A containing 0.4 M NaCl, whereas no loss of activity was observed under these conditions when 20 % (v/v) glycerol was added to the enzyme preparation (results not shown).

Molecular mass and subunit composition

Silver-stained SDS-PAGE gels of purified urease revealed three bands, corresponding to three subunits of 66 kDa (α), 18 kDa (*L. boryana*; β) or 14 kDa (*Anabaena/Nostoc*; β) and 11 kDa (γ ; Fig. 1). This heteromeric subunit composition was observed in every purification, even when purified enzyme was subjected to a second size-exclusion and ion-exchange chromatography on Mono-Q (results not shown). This suggested that the small subunits were not impurities of the purified enzyme; however, this possibility can not be ruled out with certainty. A composition consisting of α , β and γ subunits is typical for most bacterial ureases studied so far, of both Gram-positive and Gram-negative organisms (for a recent review, see Collins & D'Orazio, 1993). Subunit molecular masses of between 63 and 73 kDa (α), 8 and 17.8 kDa (β) and 8 and 16.3 kDa (γ) have been observed in other ureases (Mobley & Hausinger, 1989; Hu *et al.*, 1990; Christians *et al.*, 1991), and the subunit molecular masses of the cyanobacterial ureases studied here are in accordance with these values. However, these two ureases differ in subunit structure from cyanobacterial ureases previously described. Although the molecular masses of the holoenzymes from *Spirulina maxima* (232 kDa; Carvajal *et al.*, 1982), *Anabaena doliolum* (228 kDa; Rai, 1989) and *A. cylindrica* PCC 7122 (197 kDa; Argall *et al.*, 1992) are similar to the cyanobacterial ureases studied here, the enzymes of the three former species were described as being hexameric, composed of identical subunits with a molecular mass of between 32 and 38 kDa. In these earlier studies, however, urease was only enriched to specific activities of between 1.7 and 10 U (mg protein)⁻¹, which might suggest that these enzymes were not purified to homogeneity.

The molecular mass of the native ureases from *L. boryana* and *Anabaena/Nostoc* was determined by size-exclusion chromatography to be 230 kDa and 240 kDa respectively. In silver-stained native gradient PAGE, major protein bands corresponding to 220 kDa were observed (Fig. 1). A faint band corresponding to a molecular mass of 66 kDa was always observed (Fig. 1), which may be the α subunit, a product of a disintegration of urease into its subunits. Due to their low molecular mass, the smaller subunits (β , γ) would have passed through the separation gel. When the same gel was stained for urease activity, only one band was observed, corresponding to a molecular mass of 220 kDa (Fig. 1). This result differs from those obtained with a number of non-cyanobacterial ureases, where multiple bands of urease activity were

observed in native PAGE (Mobley & Hausinger, 1989; Schäfer & Kaltwasser, 1994). This observation was assumed to be due to disintegration of the holoenzyme into catalytically active substructures with different subunit stoichiometry.

In conclusion, we have demonstrated that the ureases from *L. boryana* PCC 73110 and *Anabaena/Nostoc* PCC 7120 are heat-stable enzymes, composed of α , β and γ subunits similar to other bacterial ureases. Our results differ from those reported for other cyanobacteria (Carvajal *et al.*, 1982; Rai, 1989; Argall *et al.*, 1992), and studies with strains belonging to various other genera of cyanobacteria are being carried out in our laboratory to clarify this aspect.

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