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Carbonic anhydrase impairment in cadmium-treated *Ceratophyllum demersum* L. (free floating freshwater macrophyte): toxicity reversal by zinc

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The influence of Cd (non-essential) and Zn (essential) on the functioning of carbonic anhydrase was investigated. Zinc restored the cadmium impaired carbonic anhydrase in *Ceratophyllum demersum* L. (coontail), a free floating freshwater macrophyte. Evidence from purified carbonic anhydrase in a two-step process using diethylaminoethyl (DEAE) Sephadex A50 and Sephadex G-25 (m = 43 kDa by SDS-PAGE), and Zn content by flame atomic absorption spectrometry, are presented.

Toxic heavy metal contamination of aquatic ecosystems is a cause of serious environmental concern for the health of biota. Cadmium (Cd) is a non-essential and toxic element, without any metabolic significance, whereas zinc (Zn) is an important component of many vital enzymes having a catalytic, co-catalytic or structural role,[1] structural stabilizers for proteins, membrane and DNA-binding proteins (Zn-fingers),[2–4] but toxic in high concentrations. Both these elements have similar geochemical and environmental properties. Non-ferrous metal production processes and subsequent release of zinc to the environment is normally accompanied by cadmium environmental pollution because of zinc ores (ZnS) generally containing 0.1–5%, and sometimes even more, cadmium.[5] The trace elements concerned are available to plants mainly from sludge-borne heavy metal applications and industrial wastewater irrigation, and partially from aerial deposition and the use of fertilizers and pesticides. This association of Cd and Zn in the aquatic environment and their chemical similarity can lead to interaction between cadmium and zinc.

Aquatic plants are known to accumulate heavy metals.[6] *Ceratophyllum demersum* is known to accumulate heavy metals and radionuclides,[7–9] especially Cd at low concentrations (0.1–0.5 ppm[10–12]). Interactions between Cd and Zn and their transfer in a soil–crop system under actual field conditions, in solution culture experiments, have been reported recently.[13,14] However, there is no information on Zn restored carbonic anhydrase in Cd exposed aquatic plants and the importance of carbonic anhydrase in *C. demersum*.

Carbonic anhydrase (CA; carbonate hydrolyase, E.C. 4.2.1.1) is a ubiquitous enzyme among living organisms which catalyses the reversible interconversion of CO_2 and HCO_3^-. CA have been extensively studied in many organisms from cyanobacteria[15,16] to higher plants,[17,18] animals and human systems.[19,20] CA has been presumed to have an active function in photosynthetic organisms[21] and has been the topic of interest in many aquatic systems where the availability of CO_2 is less (the predominant form of dissolved inorganic carbon being HCO_3^-) and further complicated by its slower diffusion rates in water, being 1×10^4 times slower than air.[22] To maintain the photosynthetic efficiency in spite of low CO_2 availability the carbon-concentrating mechanism (CCM) functions.[23,24] This CCM has two components, namely a mechanism for taking up HCO_3^- and a Zn-requiring CA that catalyses interconversion of HCO_3^- to CO_2.[25] In aquatic angiosperms it has been suggested that HCO_3^- diffusion and then conversion to CO_2 by CA plays a very vital role in maintaining the inorganic carbon levels.[26] Zn has a catalytic role in plant and animal CA, being coordinated to the imidazole rings of three histidines close to the active site.[27,28] In an earlier investigation we demonstrated that zinc alleviates cadmium-induced toxicity by using *C. demersum* as an experimental system. Since CA activity being affected by Zn deficiency[29,30] and its regulation by Zn[23] in different systems have been reported, we used CA to examine the mode of interaction between zinc and cadmium and analyze the role of Zn in CA in *C. demersum*.

Experimental

Plant material

Ceratophyllum demersum L. plants were collected from local ponds and maintained under laboratory conditions in aquaria in 1/10 Hoaglands solution, a nutrient solution containing a combination of macro- and micronutrients essential for plant growth.[31]

Cadmium and zinc treatment

Plant material (2 g) was transferred to a 250 ml glass beaker with 200 ml of 1/10 Hoaglands solution (containing 0.764 nmol of Zn) and the chosen concentration of the metals. Cd treatment of 10 μM (using $CdCl_2$) concentration was given to the plants. Zn supplementation (10, 50, 100, and 200 μM) was carried out using $ZnCl_2$ on the Cd (10 μM) treated plants. Plants were also treated with only Zn (10, 50, 100, and 200 μM) for comparison. The control plant has 57 μg g^{-1} DW Zn content in it, as reported earlier by us.[13] All the above experimental plant material was kept under the natural day and night cycle for one week. Only four treatments (Control, 10 μM Cd, Cd 10 μM + Zn 200 μM and Zn 200 μM) were chosen for purification process.

Extraction and assay of carbonic anhydrase

The treated plant material (1 g) was ground in an ice-cold mortar and pestle with 50 mM Tris-HCl buffer pH 7.5, 40 mM PMSF (phenylmethyl sulfonyl fluoride) and 2% (m/v) PVP (polyvinyl pyrrolidone). The homogenate was filtered through two layers of muslin cloth to remove the cell debris and centrifuged at 15 ,000 × *g* for 20 min and the supernatant was used as the source of the enzyme. The amount of protein was estimated according to Lowry *et al.*.[32] The assay was performed according to Armstrong *et al.*.[33] This assay is based on the

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principle that carbonic anhydrase not only catalyses the hydration of CO_2 and dehydration of HCO_3^-, but also the hydrolysis of many esters and aldehydes. This assay utilizes *p*-nitrophenyl acetate as the ester for assaying the hydrolytic reaction. 54.3 mg of *p*-nitrophenyl acetate was dissolved in 3 ml of acetone and Millipore ultrapure water was added slowly with constant stirring to a final volume of 100 ml. This solution was prepared fresh everyday. In a quartz cuvette, 1 ml of this solution was mixed with 1.7 ml of water, 0.3 ml of 0.1 M diethyl malonate was added with rapid stirring and the solution incubated for 2½ min. The increase in absorbance at 348 nm was recorded in a double beam spectrophotometer (Cintra 5, GBC Scientific, Australia) after the addition of 100 μg of protein. The activity was calculated using a molar absorptivity of 5.4 mM^{-1} cm^{-1}.

Purification of carbonic anhydrase

All the operations were carried out at 4 °C. We have followed a modified version of the procedure of Atkins *et al.*,[17] Armstrong *et al.*,[33] and Guliev *et al.*[34] Plant material (60 g) was washed thoroughly and homogenized in ice-cold buffer—50 mM Tris-HCl pH 8.7, 40 mM PMSF and 2% (m/v) PVP. The homogenate was filtered through two layers of muslin cloth to remove the cell debris and centrifuged at 15000 × *g* for 20 min. The pellet was discarded and the supernatant was used as the enzyme source. Diethylaminoethyl (DEAE) Sephadex A 50 (Sigma, USA) was washed thoroughly according to the manufacturer's instructions and packed to give a column of 2 × 35 cm. The column was equilibrated with three column volumes of 50 mM Tris-HCl pH 8.7 buffer and the enzyme source was loaded onto the column with a flow rate of 20 ml h^{-1}. After two column washings the bound protein was eluted with 0.1 M Tris-HCl, pH 8.7, and collected in 2 ml fractions. The eluate was tested for protein content by checking the positive absorbance at 280 nm (ultraviolet absorbance specific for column eluates) and CA activity and the positive fractions were pooled and concentrated by ammonium sulfate precipitation (80% saturation). The concentrate was dialysed against 3 volumes of 0.1 M Tris-HCl, pH 8.7, overnight under cold conditions. This concentrated enzyme was chromatographed on a Sephadex G-25 (Sigma, USA) column (1.5 × 35 cm) equilibrated with 0.1 M Tris-HCl pH 8.7 at a flow rate of 4.5 ml h^{-1}. One ml fractions were collected and tested for protein content by checking the positive absorbance at 280 nm (ultraviolet absorbance specific for column eluates) and CA activity. The activity rich fractions were pooled and stored in the presence of sulfhydryl agents (15 mM dithiothreitol) and referred to as the purified carbonic anhydrase.

SDS–polyacrylamide gel electrophoresis

Samples of the crude extract as well the DEAE Sephadex elution fractions and purified carbonic anhydrase from Sephadex G-25 fractions were subjected to sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as described by Laemmli,[35] to confirm the homogeneity of the purified enzyme and to estimate its molecular weight.

Zn and Cd content in purified carbonic anhydrases by FAAS

The purified carbonic anhydrase was analysed for Zn in a GBC flame atomic absorption spectrometer in an air–acetylene flame system (GBC 932 Plus, Australia). The purified sample was also analysed for Cd to verify whether there is any substitution of Zn by Cd.

Statistics

The values are means of three individual experiments with duplicates (value averaged to one) for each experiment and the results were subjected to statistical analysis by Student's *t*-test ($n = 3$). The level of significance (α) was set at 0.01 ($p \leqslant 0.01$).

Results

The results of our investigation demonstrated that Zn supplementation to a Cd poisoned plant restored CA functions, as reflected by the changes in its activity, recovery of active purified enzyme and Zn content in *C. demersum*. Further, we have also observed chlorosis in Cd 10 μM alone treatments, with the stem and leaf tissue becoming mucilagenous due to tissue degeneration. These symptoms were not observed in Zn supplemented Cd treatments. However, Zn treatments above 200 μM were found to be toxic to the plant. Zn alone treatments did not show any visible symptoms of toxicity indicating the non-toxic nature of the Zn concentrations chosen.

Carbonic anhydrase (CA) activity

The results of the assay of CA revealed a drastic 50% reduction in the activity of this enzyme in Cd 10 μM alone treated plants when compared to the control plants (Fig. 1). The activity was not only restored (67%) completely by Zn in Cd-treated plants with supplemented Zn, but it was also increased (34%) beyond the control level. However, plants treated with only Zn showed a slight increase in CA activity (Fig. 1), but not up to the levels of Cd + Zn 200 μM treatments.

Purification of carbonic anhydrase

The purification profile of CA of each treatment is shown in Tables 1–4 and the elution profiles are shown in Figs. 2–5. There was a 8.5-fold purification of the enzyme from the soluble plant protein in all the treatments. There were large differences only in the recovery of the active enzyme from each treatment. Control plants (without any metal treatment) showed a 32% recovery of the active purified protein (Table 1). Cd-treated plants showed toxicity to the enzyme as seen in the form of a very low recovery (22%) of the purified active form (Table 2). Zn supplementation to Cd-treated plants proved to be beneficial as a highly active CA with a higher recovery of 43% was purified (Table 3). Treatments with only Zn also did not affect the enzyme activity and its purification (Table 4) and a 33% recovery was observed for the purified enzyme.

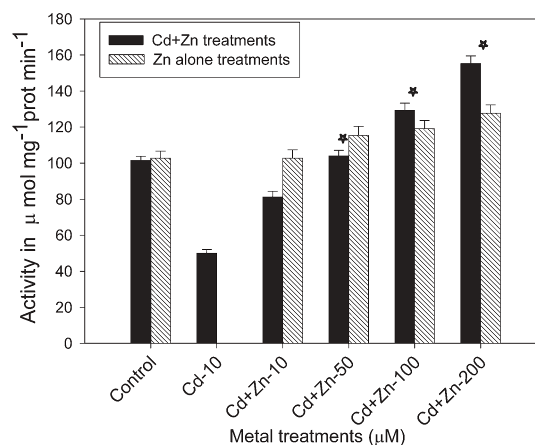

Fig. 1 Carbonic anhydrase activity in *Ceratophyllum demersum* L. treated with 10 μM Cd and Cd + Zn (10, 50, 100 and 200 μM). Error bars represent standard errors and an asterisk specifically indicates significant differences of Cd + Zn treatments from 10 μM Cd-alone treatments. Note the Cd-induced decrease in CA activity, being restored by supplementing Zn to Cd treatments.

Table 1 Purification profile of carbonic anhydrase from control plants of *Ceratophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ μmol ml^{-1}	Volume of the protein/ml[a]	Total protein[b]	Total activity[c]	Specific activity[d]/ μmol mg^{-1} protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	5	59	60	300	3540	11.8	100	1
Pooled "active" fractions from DEAE Sephadex	1.8	65	30	54	1890	36.11	53.4	3.06
Pooled "active" fractions from Sephadex G-25	0.77	77	15	11.55	1155	100	32.5	8.47

[a] Denotes the amount of crude *C. demersum* protein extract initially used as the starting material to purify CA. [b] Total protein refers to the total protein content of the entire volume of crude protein extract used. [c] Total activity refers to the CA activity of the entire volume of crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. [e] Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

Table 2 Purification profile of carbonic anhydrase from Cd 10 μM treated plants of *Ceratophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ μmol ml^{-1}	Volume of the protein/ml[a]	Total protein[b]	Total activity[c]	Specific activity[d]/ μmol mg^{-1} protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	3.2	25	60	192	1500	7.81	100	1
Pooled "active" fractions from DEAE Sephadex	1.27	30	24	30.48	720	23.62	48	3.02
Pooled "active" fractions from Sephadex G-25	0.58	38	9	5.22	342	65.51	22.8	8.38

[a] Denotes the amount of crude *C. demersum* protein extract initially used as the starting material to purify CA. [b] Total protein refers to the total protein content of the entire volume of crude protein extract used. [c] Total activity refers to the CA activity of the entire volume of crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. [e] Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

Table 3 Purification profile of carbonic anhydrase from Cd + Zn 200 μM treated plants of *Ceratophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ μmol ml^{-1}	Volume of the protein/ml[a]	Total protein[b]	Total activity[c]	Specific activity[d]/ μmol mg^{-1} protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	6.7	85.88	60	402	5152.8	12.81	100	1
Pooled "active" fractions from DEAE Sephadex	3.0	116.81	35	105	4088.35	38.93	79.35	3.03
Pooled "active" fractions from Sephadex G-25	1.15	125.92	18	20.7	2266.56	109.49	43.5	8.54

[a] Denotes the amount of crude *C. demersum* protein extract initially used as the starting material to purify CA. [b] Total protein refers to the total protein content of the entire volume of crude protein extract used. [c] Total activity refers to the CA activity of the entire volume of crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. [e] Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

Table 4 Purification profile of carbonic anhydrase from Zn 200 μM treated plants of *Ceratophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ μmol ml^{-1}	Volume of the protein/ml[a]	Total protein[b]	Total activity[c]	Specific activity[d]/ μmol mg^{-1} protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	5.8	70.37	60	348	4222.2	12.13	100	1
Pooled "active" fractions from DEAE Sephadex	2.1	77.77	32	67.2	2488.64	37.03	59	3.05
Pooled "active" fractions from Sephadex G-25	0.87	88.88	16	13.92	1422.08	102.16	33.7	8.42

[a] Denotes the amount of crude *C. demersum* protein extract initially used as the starting material to purify CA. [b] Total protein refers to the total protein content of the entire volume of crude protein extract used. [c] Total activity refers to the CA activity of the entire volume of crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. [e] Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

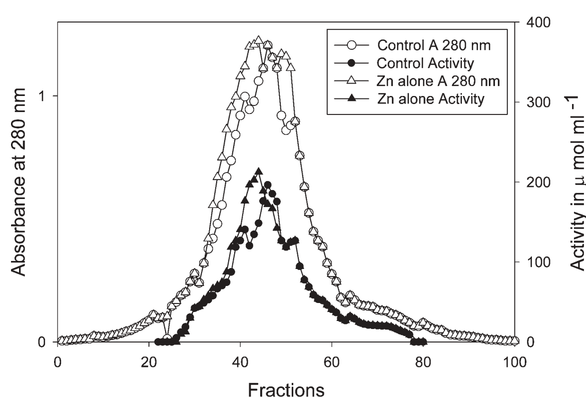

Fig. 2 Elution profile of control and Zn-alone (Zn 200 μM) treated *C. demersum* fractions from DEAE Sephadex column. The active fractions were pooled for the next purification step. Note the slight increase in activity of CA in Zn-alone treatments compared with control indicating the non-toxic and catalytic role of Zn in CA activity.

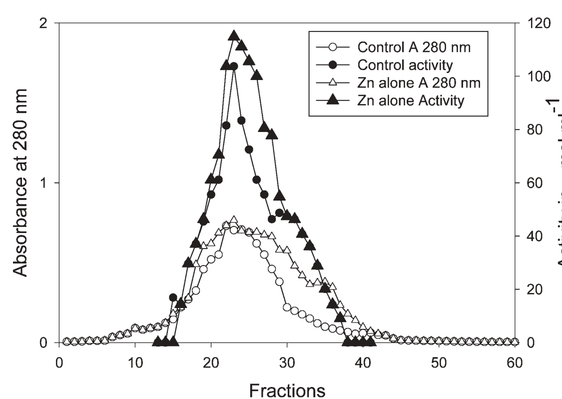

Fig. 3 Elution profile of control and Zn-alone (Zn 200 μM) treated *C. demersum* fractions from Sephadex G 25 column. The active fractions were pooled. Note the high increase in activity of purified CA in Zn alone treatments compared with control indicating the catalytic role of Zn in enhancing CA activity.

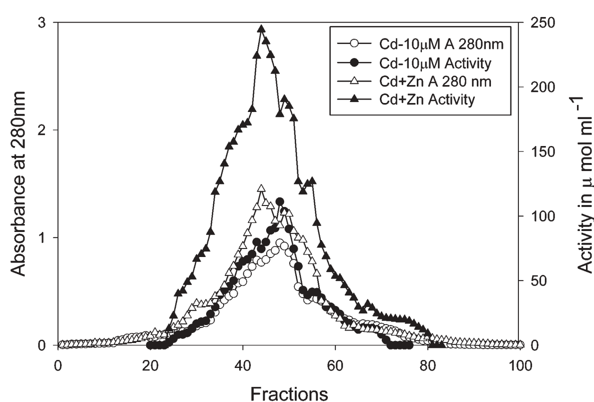

Fig. 4 Elution profile of 10 μM Cd treated and Cd + Zn 200 μM treated *C. demersum* fractions from DEAE Sephadex column. The active fractions were pooled for the next purification step. Note the drastic decrease in activity of CA in Cd-alone treatments and the restoration of CA activity in Cd treatments with supplemented Zn indicating the catalytic role of Zn in CA.

SDS-PAGE of the purified protein

Electrophoresis on a 13% gel revealed the homogeneity of the purified enzyme and the molecular weight of purified CA was 43 kDa (Fig. 6), with an additional closely associated isozyme stained as a second band very near to the purified enzyme.

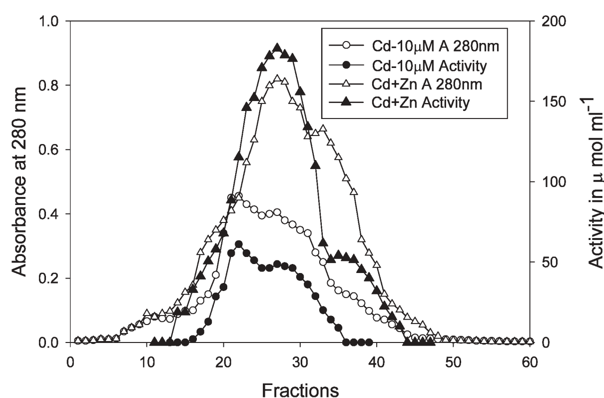

Fig. 5 Elution profile of 10 μM Cd treated and Cd + Zn 200 μM treated *C. demersum* fractions from Sephadex G-25 column. The active fractions were pooled. Note the drastic decrease in activity of CA in Cd-alone treatments and the restoration of CA activity in Cd treatments with supplemented Zn, indicating the catalytic role of Zn in enhancing CA activity.

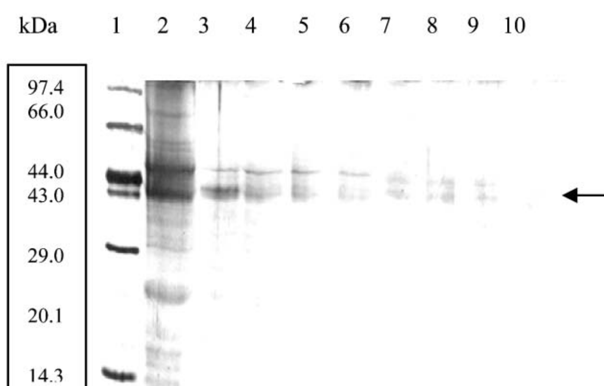

Fig. 6 13% SDS PAGE showing the protein profile at different stages of purification. Lane 1: molecular weight markers; lane 2: crude extract; lanes 3–6: DEAE Sephadex elution fractions of control, Cd 10 μM, Cd + Zn 200 μM and Zn 200 μM; lanes 7–10: Sephadex G-25 elution fractions showing the purified 43 kDa carbonic anhydrase in the same order as above.

Zn and Cd content of the purified CA

Estimation of the metal content in the purified form of CA revealed interesting results. Zn content was drastically reduced in Cd-alone treated plants showing a 73% reduction in the Zn content accounting for the impaired CA activity in Cd-treated plants. A very important observation found in our results was the detection of trace amounts of Cd (0.028 $\mu g\ mg^{-1}$ protein) in CA purified from Cd-treated plants (Fig. 7). In contrast, in Cd-treated plants with supplemented Zn there was a total restoration of the Zn content (77%) as well as a slight increase in Zn content indicating the nature of Zn in overcoming Cd toxicity. Zn-alone treated plants, however, did not show differences in the Zn content from the CA purified from control. Except in CA purified from Cd-treated plants there was no trace detection of Cd in other treatments.

Discussion

This study was undertaken to identify the mechanism of interaction between Cd and Zn with CA as the enzyme, an important enzyme in aquatics for bicarbonate assimilation. Our earlier investigations have shown that Zn alleviates Cd-induced oxidative stress by its antioxidative capacity through the influence of antioxidant enzymes.[13] The present investigation revealed the existence of active Zn-dependent CA in

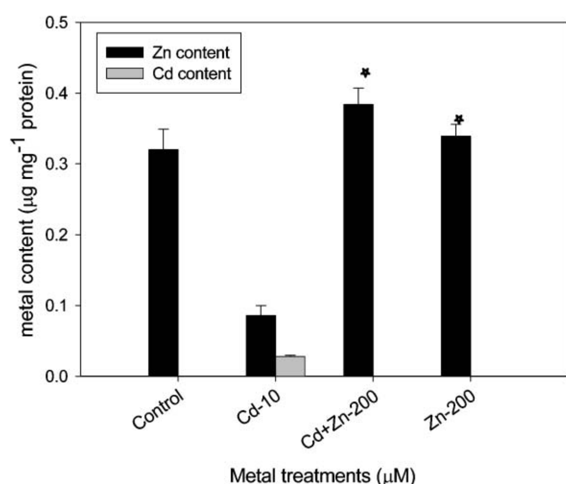

Fig. 7 Zn and Cd content in purified carbonic anhydrase from control, 10 μM Cd, Cd + Zn 200 μM and Zn 200 μM treatments. Error bars represent standard errors and an asterisk specifically indicates significant differences from 10 μM Cd treatments. It can be inferred that traces of Cd detected, substituting Zn in the active site of CA, were efficiently removed and restored by Zn supplements to Cd treatment.

C. demersum like other aquatic macrophytes, although its location, whether periplasmic or intracellular, is yet to be investigated.[26] The results of our experiments indicated that CA is impaired by Cd (Fig. 1). Cadmium (like several other heavy metals) is known to affect the structure and functioning of enzymes[36] through peroxidation and production of toxic reactive oxygen species (ROS),[37] which would affect the functioning of the enzymes and proteins. ROS induce fragmentation of protein and impose oxidative modification, rendering cells susceptible to enzymatic proteolysis and hydrolysis.[38] However, Cd-treated plants, when supplemented with Zn, showed not only active restoration of the CA activity, but also an increase in activity at the highest concentration of Zn, demonstrating the complete dependence of the active form of this enzyme on the presence of sufficient levels of Zn (Fig. 1). Zn does not undergo oxidation or reduction reactions and hence is stable in the biological medium, whose oxidoreductive potential is subject to continous flux.[39] This property of Zn helps in maintaining the chemical potential of the Zn enzymes and associated proteins, protecting them from peroxidative damage and oxidative stress.[13]

Cd, Hg and Zn form the group II transition metals with eight electrons filled in their outer orbital. Since Cd and Zn, both taken as divalent cations, are similar in configuration, Cd can readily inhibit most of the Zn-dependent processes, either by displacement or by occupying the active sites of the Zn metalloproteins.[40,41] The purification of CA (Fig. 6) from *C. demersum* revealed this toxic function of Cd towards CA. There was a reduction in the recovery as well the activity of the enzyme purified from Cd 10 μM-alone treated plants (Table 2, Figs. 4 and 5). Thus, the theory of Cd occupying the active sites of important Zn–metalloproteins has been proved by our findings. The estimation of Zn content in the purified enzyme not only showed a drastic reduction in the amount of Zn but also traces of Cd (Fig. 7), as recorded by flame atomic absorption spectrometry. Our experiments showed a reduction of 73% in Zn content and its substitution by Cd impaired the structure as well as the activity of CA. This will lead to non-functioning of CA and hence the associated photosynthetic processes also will be impaired. In many cases it has been reported that removal of Zn from CA leads to irreversible loss of catalytic activity,[42] which corroborates our results. Zn being catalytically associated with CA, maintains the spatial relationships with the different amino acid residues in the polypeptide backbone and maintains the protein conformation intact.[27] In such a case even a slight change in the protein conformation by substitution of catalytically inactive Cd would cause distorted unfoldings to form a totally impaired structure.[2]

Redox active metals like Cu and Cd are known to primarily affect the enzymes and proteins through its interaction with the –SH groups and induce redox cycling.[43] Probably, the Zn–thiolate bonds of the enzyme are broken and replaced by disulfide groups by an inactive and toxic metal like Cd.

The CA purified from Cd-treated plants with supplemented Zn showed a higher recovery as well as highly active CA (Table 3, Figs. 4 and 5). The Zn content estimated was also slightly higher than that of the control, accounting for the increased uptake of Zn for proper functioning of CA (Fig. 7). Zn-alone treatments also showed an active enzyme purified with Zn content closely comparable with that of the control (Table 4, Figs. 2, 3). The presence of sufficient levels of Zn in Cd-treated plants with supplemented Zn strengthens the Zn–metalloprotein interaction by protection of the –SH groups from thiol oxidation and intramolecular disulfide formation, a function primarily associated with Zn.[44] Zn, by associating with the –SH groups, renders them inactive by steric hindrance and hence their susceptibility towards disulfide formation.[45] Zn is a versatile interactant with the enzymes and has a variable coordination sphere and stereochemical adaptation to assume multiple coordination geometries, contributing to its unique and efficient functioning in the metalloenzymes.[39,46] In conclusion, Zn restored the Cd impaired CA in *C. demersum*.

Acknowledgements

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References

1 I. Cakmak and H. J. Braun, in *Application of Physiology in Wheat Breeding*, eds. M. P. Reynolds, J. I. Ortiz-Monasterio and A. McNab, D.F.CIMMYT, 2001, pp. 183–199.
2 B. L. Vallee and D. S. Auld, *Biochemistry*, 1990, **29**, 5647–5659.
3 M. N. V. Prasad, *Zeszyty Naukowe PAN*, 2002, **33**, 363–356.
4 M. N. V. Prasad, *Zeszyty Naukowe PAN*, 2002, **33**, 49–54.
5 *Trace Elements in Terrestrial Environments: Biogeochemistry, Bioavailability, and Risks of Metals*, ed. D. C. Adriano, Springer-Verlag, New York, 2nd edn., 2001, p. 866.
6 M. N. V. Prasad, M. Greger and N. Smith Bruce, in *Metals in the Environment: Analysis by Biodiversity*, ed. M. N. V. Prasad, Marcel Dekker, New York, 2001, pp. 259–288.
7 A. I. A. Bolsunovskii, A. I. Ermakov, M. Burger, A. G. Degermendzhi and A. I. Sobolev, *Radiat. Biol. Radioecol.*, 2002, **42**, 194–199.
8 A. Szymanowska, A. Samecka-Cymerman and A. J. Kempers, *Ecotoxicol. Environ. Saf.*, 1999, **43**, 21–29.
9 P. Gupta and P. Chandra, *Waste Manage.*, 1996, **16**, 335–337.
10 W. H. Ornes and K. S. Sajwan, *Water, Air, Soil Pollut.*, 1993, **69**, 291–300.
11 G. Pavankumar and M. N. V. Prasad, *Bull. Environ. Contam. Toxcicol.*, 2003, in the press.
12 G. Pavankumar and M. N. V. Prasad, *Bull. Environ. Contam. Toxcicol.*, 2003, in the press.
13 P. Aravind and M. N. V. Prasad, *Plant Physiol. Biochem.*, 2003, **41**, 391–397.
14 Z. Nan, J. Li, J. Zhang and G. Cheng, *Sci.Total Environ.*, 2002, **285**, 187–195.
15 K. Aizawa and S. Miyachi, *FEMS Microbiol. Rev.*, 1986, **39**, 215–233.
16 S. Smith Kerry and G. Ferry James, *FEMS Microbiol. Rev.*, 2000, **24**, 335–366.
17 C. A. Atkins, B. D. Patterson and D. Graham, *Plant Physiol.*, 1972, **50**, 214–217.

18 D. Graham, M. L. Reed, B. D. Patterson, D. G. Hockley and M. R. Dwyer, *Ann. NY Acad. Sci.*, 1984, **429**, 222–237.
19 E. Egon, S. A. Rickli, S. Ghazanfar, H. B. Gibbons and J. T. Edsall, *J. Biol. Chem.*, 1964, **239**, 1065–1078.
20 C. Brian, K. Tripp, K. Smith and J. G. Ferry, *J. Biol. Chem.*, 2001, **276**, 48615–48618.
21 D. Sultmeyer, C. Schmidt and H. P. Fock, *Physiol. Plant.*, 1993, **88**, 179–190.
22 M. R. Badger, in *The Biochemistry of plants: A comprehensive treatise, Volume X*, eds. M. D. Hatch and N. K. Boardman, Academic Press, New York, 1987, pp. 219–274.
23 T. W. Lane and F. M. M. Morel, *Plant Physiol.*, 2000, **123**, 345–352.
24 N. Rascio, *Crit. Rev. Plant. Sci.*, 2002, **21**, 401–427.
25 M. R. Badger and G. D. Price, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1994, **45**, 369–392.
26 J. T. M. Elzenga and H. B. A. Prins, *Aust. J. Plant Physiol.*, 1988, **15**, 727–735.
27 Y. Pocker and S. Sarkanen, *Adv. Enzymol.*, 1978, **47**, 149–274.
28 J. E. Lamb, *Life Sci.*, 1977, **20**, 393–406.
29 Z. Rengel, *Plant Physiol.*, 1995, **147**, 251–256.
30 P. J. Randall and D. Bouma, *Plant Physiol.*, 1973, **52**, 229–232.
31 D. I. Arnon, *Am. J. Bot.*, 1938, **25**, 322–325.
32 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **192**, 265–275.
33 J. McD. Armstrong, D. V. Myers, J. A. Verpoorte and J. T. Edsall, *J. Biol. Chem.*, 1966, **241**, 5137–5149.
34 N. M. Guliev, G. G. Babaev, S. M. Bairamov and D. A. Aliev, *Russ. J. Plant Physiol.*, 2003, **50**, 213–219.
35 U. K. Laemmli, *Nature*, 1970, **277**, 680–685.
36 F. Van Assche and H. Clijsters, *Plant Cell Environ.*, 1990, **13**, 195–206.
37 K. J. Dietz, M. Baier and U. Kraner, in *Heavy Metal Stress in Plants—From Molecules to Ecosystems*, eds. M. N. V. Prasad and J. Hagemeyer, Springer-Verlag, Heidelberg, 1999, pp. 73–98.
38 S. P. Wolff, A. Garner and R. Dean, *Trends Biochem. Sci.*, 1986, **11**, 27–31.
39 B. L. Vallee and K. H. Falchuk, *Phys. Rev.*, 1993, **73**, 79–118.
40 E. Nieboer and D. H. S. Richardson, *Environ. Pollut. (Ser. B)*, 1980, **1**, 2–26.
41 A. Siedlecka, *Acta Soc. Bot. Pol.*, 1995, **3**, 265–272.
42 N. M. Guliev, Sh. M. Bairamov and D. A. Aliev, *Sov. Plant Physiol.*, 1992, **39**, 537–544.
43 S. R. Powell, *J. Nutr.*, 2000, **130**, 1447s–1454.
44 I. Cakmak, *New Phytol.*, 2000, **146**, 185–205.
45 T. M. Bray and W. J. Bettger, *Free Radicals Biol. Med.*, 1990, **8**, 281–291.
46 J. E. Coleman, *Curr. Opin. Chem. Biol.*, 1998, **2**, 222–234.