BioMetals **14:** 85–94, 2001.

Earthworm pre-procarboxypeptidase: a copper responsive enzyme

S.R. Stürzenbaum*, S. Cater, A.J. Morgan & P. Kille
School of Biosciences, University of Wales, P.O. Box 911, Cardiff CF1 3US, Wales, UK
**Author for correspondence (Tel: +44 2920 874119; Fax: +44 2920 874116; E-mail: SturzenbaumSR@Cardiff.ac.uk)*

Received 23 January 2001; accepted 29 January 2001

Key words: carboxypeptidase, heavy metal pollution, earthworms, *Lumbricus rubellus*

Abstract

This paper describes the identification and molecular cloning of the earthworm metalloenzyme pre-procarboxypeptidase. It was possible to approximate the putative pre-pro cleavage sites, which after removal activates a 35.7 kDa mature peptide with a length of 317 amino acids. The primary, secondary and predicted tertiary structure of the mature chain is highly homologous to other carboxypeptidases from diverse phylogenetic origin. Using a fully quantitative PCR, we were able to assess relative expression of this gene in earthworms exposed to different heavy metal contaminated substrates. In summary, cadmium on its own or in combination with lead and zinc, did not trigger transcriptional up-regulation of carboxypeptidase. In contrast, copper exposed earthworms were assessed to have a 4-fold increase in carboxypeptidase transcript numbers. A hypothetical model is presented to explain how the exposure to heavy metals may influence transcriptional control and/or function of this enzyme. Finally, the significance of these observations in terms of risk assessment and biomonitoring of contaminated soils is discussed.

Introduction

Carboxypeptidases form a major family of digestive enzymes responsible for the release of essential amino acids from proteins and peptides within the alimentary tract. The catalytic mechanism of these enzymes centres around a zinc ion which is tetrahedrally coordinated by a water molecule, a glutamate and two histidines (Barrett *et al.* 1998). This zinc ion is exposed to the surrounding environment and displacement by other metal ions may compromise the activity of the enzyme. For example, carboxypeptidase U activity may be inhibited by cobalt (Hendriks *et al.* 1989) or indeed stimulated by cadmium (Campbell & Okada 1989). Moreover, the substitution of zinc in lysosomal carboxypeptidase A by heavy metals such as copper, silver and mercury generates an enzyme with a markedly reduced enzymatic function (Chikuma *et al.* 1996; Itoh *et al.* 1993; Tranchemontagne *et al.* 1990). The activity of this family of enzymes is therefore dependant on their ability to retain a zinc ion within the catalytic site.

The complex regulation of metal ions within cells ensures the delivery and incorporation of specific metal ions into cytosolic metallo-proteins and prevents competition for metal binding sites (Sarkar 1999). However, when carboxypeptidases are released into the digestive system they are exposed to an environment where the metal content is tightly controlled by the dietary constituents. This phenomenon may be particularly exacerbated in soil or sediment feeders, such as the earthworms or lugworms, which by their very nature ingest large quantities of the surrounding matrix (Chen & Mayer 1998). Earthworm populations have been identified to inhabit a wide spectrum of metal contaminated environments which may contain high levels of cobalt, cadmium or copper (Morgan & Morgan 1990; Streit 1984). If these conditions effect the activity of the earthworm digestive carboxypeptidase homologues, it may impact on the absorption effi-

The sequence data reported in this paper has been submitted to the EMBL/Genbank libraries under the accession number Y09625.

ciency of amino acids from the alimentary system. It is unclear whether a negative feedback mechanism may compensate for any activity loss caused by competing heavy metals within the digestive environment.

The present study describes an investigation to identify metal responsive genes which has led to the isolation of the first earthworm carboxypeptidase gene. The expression of this gene is examined in earthworms exposed to different metal loaded environments. Furthermore, the overall architecture, catalytic components and specificity of this novel enzyme is examined by comparative molecular modelling.

Materials and Methods

Earthworms

Adult earthworms (*Lumbricus rubellus*) were sampled from a heavy metal uncontaminated site (Dinas Powys, Wales, UK, O.S. Grid reference: ST 149 723). Earthworms were either directly sacrificed for subsequent experimentation or maintained on artificial soil (made in accordance to OECD guidelines) for a 2-week acclimatization period and then transferred for a further 2 weeks to artificial soil supplemented with 250 μg $CdCl_2$ kg dry artificial soil. In addition, earthworms (same species) were sampled from spoil heaps of abandoned mines. Sites included a predominantly copper ($>$800 μg Cu g dry weight) contaminated mine (South Caradon, Wales, UK, O.S. Grid reference: ST 149 723), and a lead ($>$2300 μg Pb g dry weight), zinc ($>$5900 μg Zn g dry weight) and cadmium ($>$600 μg Cd g dry weight) contaminated mine (Rudry, Wales, UK, O.S. Grid reference: ST 149 723).

Isolation of RNA

Total RNA was extracted using Tri-Reagent (Sigma) with a subsequent poly-A RNA isolation (Pharmacia, Biotech.). Transcript quantification was performed on reverse transcribed mRNA obtained using the poly-A RNA isolation kit (Pharmacia, Biotech.). All isolations consisted of the posterior alimentary canal and its surrounding tissue from a pooled sample of 4 earthworms.

Directed differential display and RACE-PCR

A directed differential display reaction was performed using primers encoding for metal-binding motifs. The resulting differential bands were excised, re-hydrated, re-amplified, cloned and sequenced essentially as described elsewhere (Stürzenbaum *et al.* 1998a). The full length sequence was obtained by designing sense (5′-TACCTAGCGGGAACTGCTCCA-3′) and anti-sense (5′-CACCAGCCCAATTTTCAGCA-3′) primers to the fragment obtained by directed differential display and performing a RACE PCR (Clontech) utilizing a Marathon RACE cDNA mini library synthesized from earthworms native to Rudry mine (see Stürzenbaum *et al.* 1998b). PCR products were ligated into the pGEM-T vector (Promega) as recommended by the manufacturer.

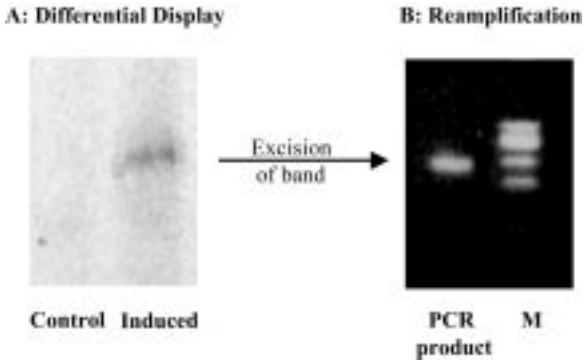


Fig. 1. Directed differential display of carboxypeptidase in the earthworm *L. rubellus*. Differential display and reamplification of carboxypeptidase. **Panel A** shows the band that was excised from the differential display gel of mRNA derived from heavy metal contaminated earthworms as described in Stürzenbaum *et al.* 1998d) The product was reamplified and subjected to electrophoresis on a 2% agarose gel, containing ethidium bromide (0.5 μg/ml). Nucleic acids were visualized under ultraviolet light (**Panel B**). M = Nucleic acid molecular weight markers (310 bp, 281 bp, 271 bp and 234 bp).

Sequencing and database searches

Recombinant plasmid clones were sequenced using the thermosequenase cycle sequencing kit (RPN 2438, Amersham) with infrared-labelled universal forward and reverse sequencing primers. Electrophoresis, band detection and data processing were performed using a Li-cor 4000LS sequencer. Sequence identity and relatedness was confirmed accessing the DNAsis analysis program (Hitachi Software) and database homology searches (BlastX and BlastN).

Modelling of tertiary structure

Protein molecular modelling was performed on a Silicon Graphics O2 R5000 workstation (Phoenix Computers Ltd.) with the InsightII suite of software (Molecular Simulations Inc.). The predicted structure of

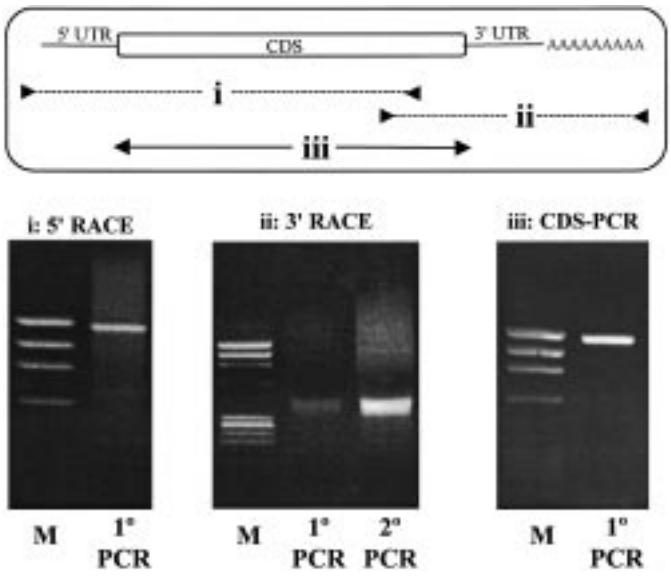


Fig. 2. Schematic diagram of the amplification strategy to obtain the gene encoding for earthworm carboxypeptidase. Indicated are the approximate positions of RACE-PCRs (5′ and 3′ RACE) as well as the final (confirmative) amplification of the entire coding region (CDS). Note that the 3′ RACE required a nested (2 °) PCR to increase the specificity of amplification. M = ϕ nucleic acid molecular weight marker (1352 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 271 bp, 234 bp).

earthworm carboxypeptidase was modelled from the known crystal structure of the bovine carboxypeptidase using the 'Homology' module and refined by applying molecular dynamics performed by 'Discover3'.

Quantitative microvolume rapid PCR

Sybr® Green's specific fluorescence, when binding to the minor groove of double stranded DNA molecules, can be exploited to monitor product formation during polymerase chain reaction (PCR). Gene specific fluorescent profiles were determined by quantitative PCR using a LightCycler™ (Idaho Technology Inc.). In addition, the specificity of each amplified end product was assessed by melting point analysis (as described in Stürzenbaum *et al.* 1998c) and agarose gel electrophoresis. Specific primers (5′-CTGTTCGCTGTGCCAAATCA-3′ and 5′-GGGTGGTGTGTGGGTGCCTCT-3′) were designed to amplify fragments avoiding mis-match and the formation of primer artifacts. Amplifications were optimised for $MgCl_2$, Sybr® Green I and variable annealing temperatures. Amplification reactions consisted of 0.5 μl cDNA template (plasmid cloned DNA preparation or reverse transcribed mRNA), 1 pM of each primer (synthesised by MWG-Biotech GmbH), 0.2 mM dNTPs, 1:30 000 dilution of Sybr® Green I and 1 unit of Taq polymerase (Promega), buffered with 50 mM Tris, 250 μg ml BSA and supplemented with 2–4 mM $MgCl_2$ (Biogene). The PCR reactions were performed in microvolume capillaries and subjected to 94 °C for 1 sec (ramp rate: 20 °C sec), 60 °C for 3 seconds (ramp rate: 20 °C sec), and 72 °C for 10 sec (ramp rate: 3 °C sec), for 40 cycles. Product formation was monitored at the end of each 10 sec extension step.

Dilutions of plasmid stocks were prepared and analysed in duplicates as described earlier (Stürzenbaum *et al.* 1998d) thus allowing the generation of a regression line by plotting cycle number needed to attain threshold fluorescence pertaining to the mid-logarithmic portion of the amplification against $\log_{10}$ [molecules of target gene]. The number of target molecules present in each reverse transcribed cDNA were quantified by regression analysis, extrapolating each 'unknown' over the standard range. All quantifications were performed in triplicates. It is also worth noting that earthworms chosen for analysis were of comparable size, weight and developmental age. Genes quantified included carboxypeptidase and actin (optimised as described previously in Stürzenbaum *et al.* 1998d).

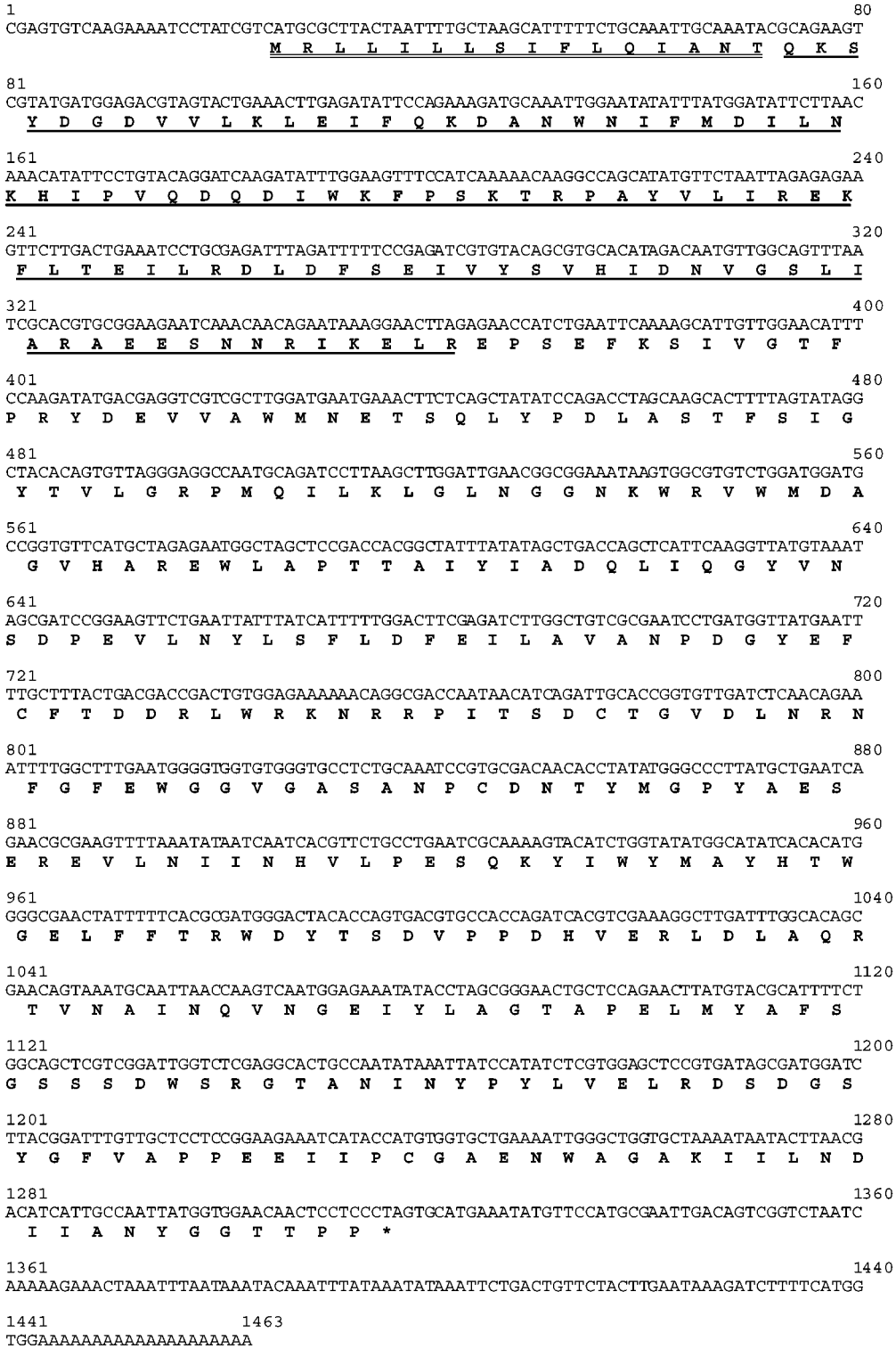


Fig. 3. The gene encoding for pre-procarboxypeptidase in the earthworm. Depicted is the base sequence with the amino acid translation of the encoded protein. Deduced from the known cleavage sites of human pre-procarboxypeptidase (Laethem *et al.* 1996) the putative regions of the pre, pro and mature peptides are indicated. The sequence was submitted to international genetic databases under accession number Y09625.

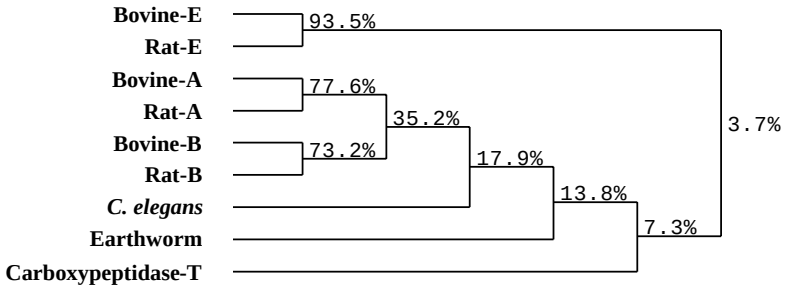


Fig. 4. Phylogenetic tree of carboxypeptidase isoforms. Sequences include human carboxypeptidase-A, B and E (*Bos taurus*, PID accession numbers g115878, g115882 and g115891 respectively), rat A, B and E (*Rattus norvegicus*, PID: g1345702, g115886 and g115893), as well as an enzyme with undefined isoform classification from nematode (*C. elegans*, PID: G3786502), bacteria (*Streptomyces capreolus*, PID: g729062) and earthworm (*Lumbricus rubellus*, PID: g2624198). Similarity scores are expressed as % homology at the amino acid level.

Results

Elucidation of nucleotide and amino acid sequence

The sequence of a small 250 bp unidentified fragment obtained from a directed differential display reaction (Figure 1A and B) was utilized to design primers to elucidate the full-length sequence of the gene described in this paper. Using an adapter ligated mini library, it was possible to determine the 5′ end, the translated coding region, as well as the 3′ untranslated region of the gene (see Figure 2 for sequencing strategy). In detail, the sequence was deduced by performing a 5′ RACE (Figure 2 i) and a 3′ RACE (Figure 2 ii). Furthermore, the overlap region was confirmed by conventional PCR, designed to amplify the coding region of the gene (Figure 2 iii). The sequence encompasses 1463 base pairs, translating into a 430 amino acid product with a molecular weight of 43.3 kDa (Figure 3). Concurrent database homology searches confirmed the successful amplification of the gene encoding for carboxypeptidase. Based solely on circumstantial evidence (Laethem *et al.* 1996), it was possible to approximate the putative pre-pro cleavage sites, which after removal activates the remaining 35.7 kDa mature peptide with a length of 317 amino acids (Figure 3). Therefore, this enzyme should be referred to (in true taxonomic terms) as an earthworm pre-procarboxypeptidase. A phylogenetic tree analysis to homologous mature carboxypeptidase protein sequences from other organisms clearly showed both the earthworm and the *C. elegans* enzymes to be distinct from the well defined subdivisions A, B and E (Figure 4).

Transcript quantification

In order to quantify carboxypeptidase expression in earthworms, a differential Southern procedure was applied, essentially as described in (Stürzenbaum *et al.* 1998c). Although the technique was performed successfully *per se* (as judged by the positive signals obtained from actin), the expression patterns of carboxypeptidase failed to show up on the autoradiography film (data not shown), thus indicating that its transcriptional message was below the detection range of ^{32}P radiolabelled quantitative approaches. In contrast, transcript quantification by PCR is far more sensitive and because of its stable and rapid amplification, it provides a powerful means of evaluating gene expression in a true quantitative manner. Indeed, it was possible to quantify the expression of carboxypeptidase in earthworms acclimated to four different soils (Figure 5A & B). Basal levels of carboxypeptidase were determined in earthworms sampled from a heavy metal unpolluted control site and measured to be $8.4 \times 10^{-1} \pm 0.2$ (molecules of carboxypeptidase per molecule of actin (an invariant control gene) in 1 μl reverse transcribed cDNA $\pm$ standard error, $N = 3$). Carboxypeptidase levels remained remarkably constant in control earthworms post-transferral to artificial soil, supplemented with 250 μg $CdCl_2$ kg dry soil as well as earthworms sampled from Rudry, the Pb, Zn and Cd contaminated mine site. Carboxypeptidase transcripts were quantified to be $8.9 \times 10^{-1} \pm 0.3$ and $7.3 \times 10^{-1} \pm 0.1$, respectively. However, a significant up-regulation was observed in earthworms native to South Caradon, the predominantly copper contaminated mine site, where carboxypeptidase levels increased, in relation to the basal levels, in excess of four fold to $30.1 \times 10^{-1} \pm 1.9$ molecules of carboxypeptidase per molecule of actin (Figure 5 C).

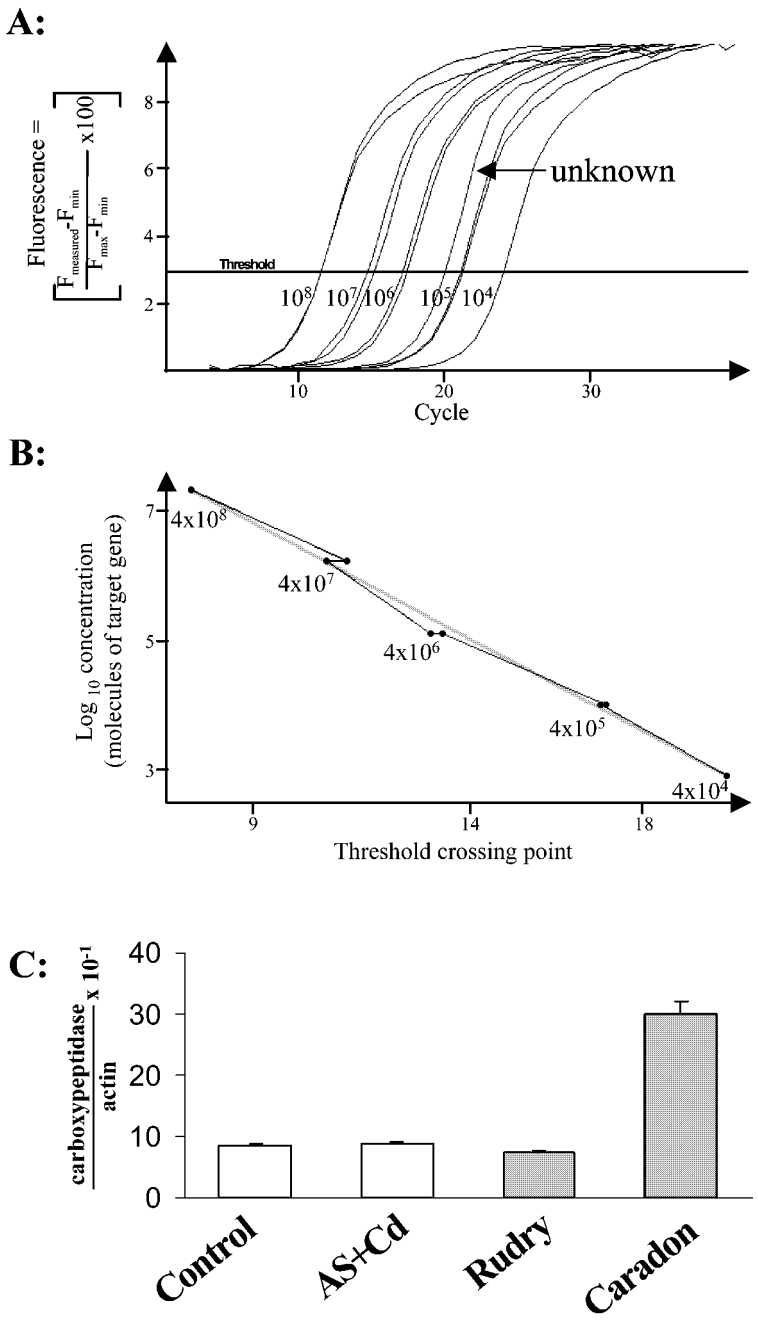


Fig. 5. Quantitative amplification of the carboxypeptidase gene. Panel A depicts the exponential amplification of a dilution series of carboxypeptidase standards (10^8 to 10^4 molecules) and one sample of cDNA with an unknown carboxypeptidase concentration. Panel B illustrates the computer-generated regression, which allows the extrapolation of the unknown concentration over the diluted series of standards and thus an accurate quantification of transcript number (Panel C). Note: The experimental analyses were performed on amplifications free of primer artifacts, including all standards (in duplicates) and all 'unknowns' (in triplicates).

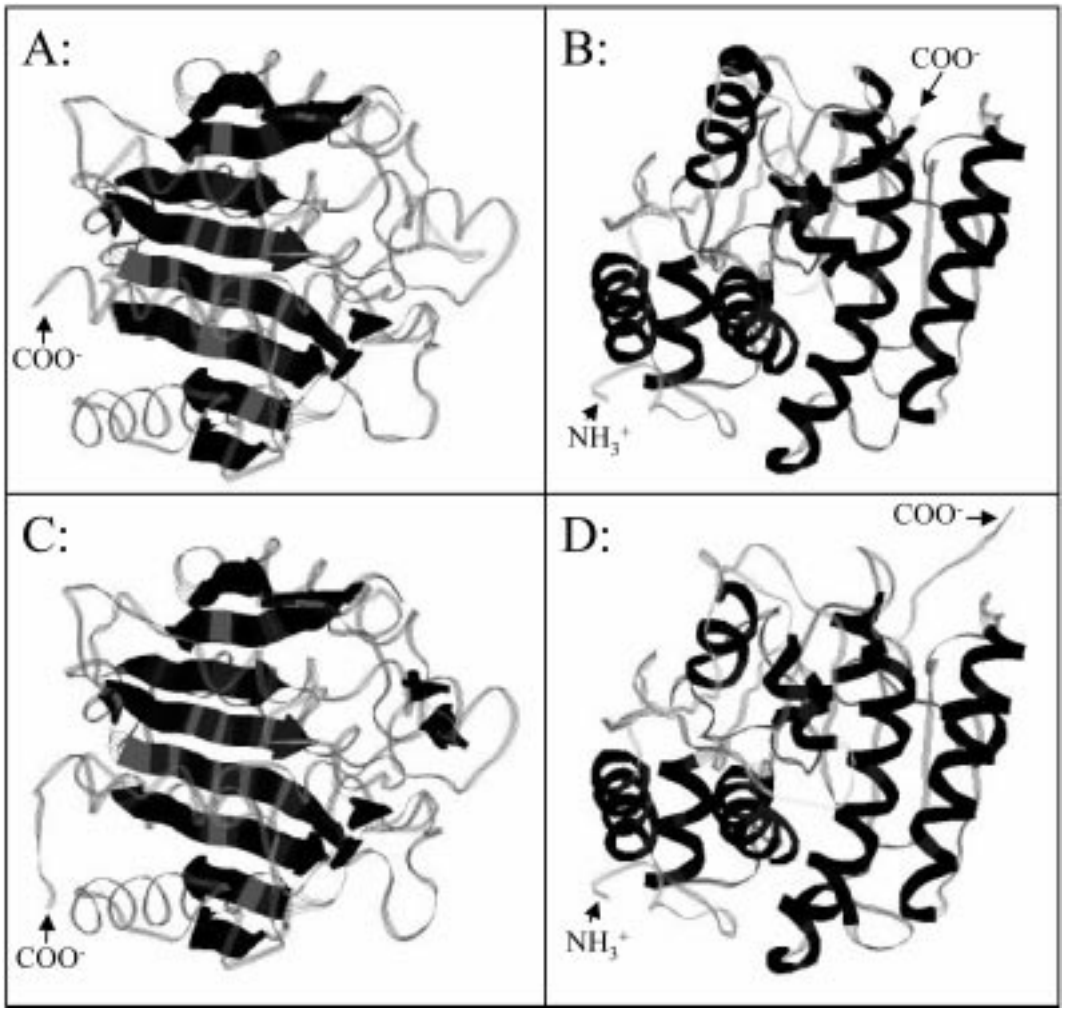


Fig. 6. Prediction of 3-dimentional tertiary structure of earthworm mature carboxypeptidase. The earthworm enzyme was modelled on the bovine (*Bos taurus*) carboxypeptidase A, previously identified by X-ray diffraction (MMDB accession number 4876). Note that earthworm α helices (Panel A) and bovine α helices (Panel B) as well as earthworm β sheets (Panel C) and bovine β sheets (Panel D) are conserved.

Prediction of tertiary structure

Based on the known crystal structure of bovine carboxypeptidase, it was possible to predict the tertiary structure of the earthworm homologue. The predicted overall architecture is very similar with all structurally significant features conserved. These entail the location and folding of the central sheet (Figure 6A and C) which is encapsulated by numerous helices (Figure 6B and D), the location of the two cysteine residues responsible for the formation of disulphide bonds as well as the zinc co-ordinating residues of the catalytic site (Figure 7A and B). The key residues of the active site (Try248 and Glu270) are conserved, however the enzyme specificity defining residues are not (Figure 8). One further significant structural difference exists, namely the enlarged C-terminal extension of the earthworm protein (Figure 6A and C).

Discussion

This paper describes the isolation, molecular cloning and initial characterization of a copper-responsive carboxypeptidase in the earthworm *L. rubellus*. Carboxypeptidases belong to the group of zinc-containing exopeptidases and are essential in the degradation of (food-) proteins resulting in the release of amino acids (Auld 1998). The enzyme is stored as an inactive precursor form and is activated by an N-terminal pro-

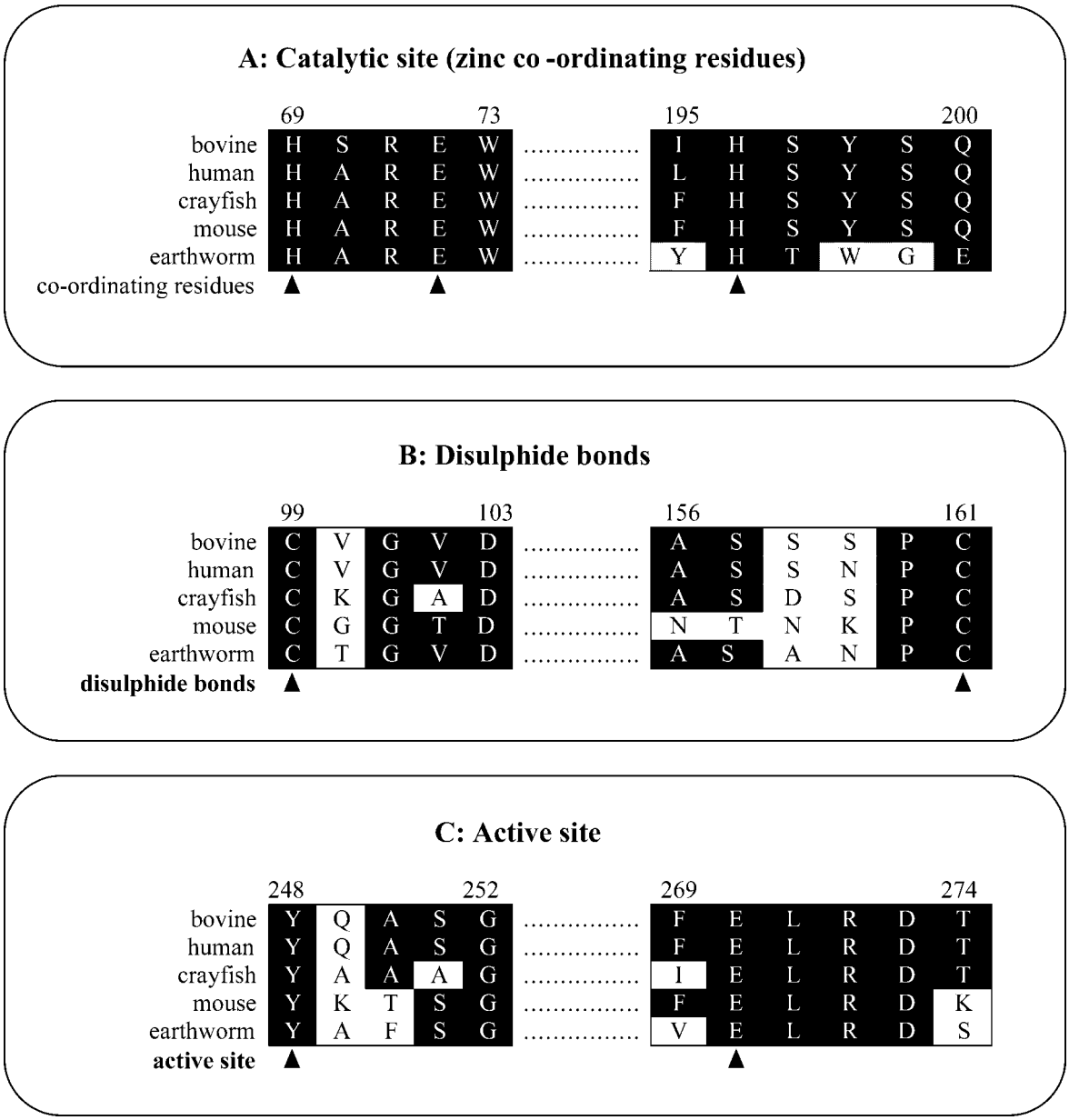
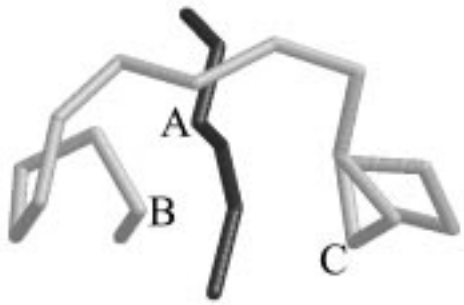


Fig. 7. Important residues of carboxypeptidases are conserved, including the zinc co-ordinating residues of the catalytic site (Panel A), the cysteine residues implicated in disulphide formation (Panel B) and the active site (Panel C). The numbering of the amino acids is based on the mature chain of bovine carboxypeptidase A.

teolytic process. The earthworm cDNA sequence is just under 1.5 kb long and encodes a pre-proenzyme of 402 amino acids, including a putative signal peptide (pre region) and activation peptide (pro region) of 16 and 97 amino acids, respectively. Notably, all amino acid residues previously implicated in enzyme function and Zn chelation (Colombo *et al.* 1995) are conserved throughout the animal phyla (see Figure 4). Given that the earthworm sequence is no exception suggests analogous structure-function relationships. The molecular modelling of the molecule, which showed an exceptional similarity to the extensively studied bovine structure, supports this assumption. Furthermore, the majority of α-helices and β-sheets are conserved, with residues involved in the Zn ion coordinating catalytic site, disulphide bond formation

and the active site of the enzyme all being perfectly conserved (Figures 6 and 7). Two notable differences can be identified, firstly the C-terminal of the earthworm 317 amino acid mature enzyme is marginally larger than its closest homologues (see Figure 6). The significance of this finding is uncertain, but given its distant proximity to the active site, it is unlikely to be involved in metal binding properties. The second aspect concerns the specificity pocket of the enzyme. Mammalian carboxypeptidase A, for example, has a wide substrate specificity but prefers peptides with aromatic or branched-chained C-terminal amino acids (Auld 1998). This specificity can be explained by the presence of Ile243 and Ile255 located on two perpendicular alpha helices with Gly207 arrange on a stand running through the cleft between the two isoleucine residues (Figure 8). In contrast, carboxypeptidase B specifically cleaves at C-terminal lysine and arginines (Folk *et al.* 1960) a specificity defined by a Gly and Asp within the two helices and a Ser in the cleft. Close examination of the earthworm model reveals an Ala and Ser located on two perpendicular alpha helices with a charged Asp situated on the underlying stand. The presence of the negatively charged amino acid highlights its similarity to carboxypeptidase B and may indicate its preference for similar lysine or arginine side chains. However, the true enzymatic function of earthworm carboxypeptidase can only be assessed by isolating the active protein, an undertaking we are currently pursuing in our laboratory. This will allow the evaluation of cleavage activity/specificity and thus allow us to derive the true functionally classification of the earthworm enzyme.

But even without this type of information, some fascinating details have been forthcoming from the quantification of carboxypeptidase transcript numbers in earthworms exposed to soils with different concentrations of diverse heavy metals. It seems that exposure to Cd does not alter transcription of carboxypeptidase. Similarly, carboxypeptidase transcription was unchanged in earthworms native to a heavily polluted mine site (Rudry), notably contaminated with Cd, Pb, but also Zn. A stark upregulation was measured in earthworms native to a copper contaminated mine site (South Caradon). Although copper is an essential metal, it is toxic in large quantities. Furthermore, the chemical stoichiometry of copper is vastly different to zinc and cadmium. It is therefore conceivable that a copper substitution of the co-factor zinc in carboxypeptidase will change the three-dimensional structure of the enzyme and thus may compromize its



	A_{207}	B_{243}	C_{255}
Bovine A :	Gly	Ile	Ile
Bovine B :	Ser	Glu	Asp
Earthworm :	Asp	Ala	Ser

Fig. 8. A schematic representation of the carboxypeptidase specificity pocket. The carbon backbone of residues 204–209 is black whilst residues 242–259 are given in grey. Indicated are the positions (A, B and C) within the protein that are implicated in enzyme specificity. Residue numbering refers to the mature bovine carboxypeptidase A protein.

dynamics or even render it inactive (Hofman 1985). As a result a transitional feedback mechanism may be invoked and the direct cause of the observed transcription of carboxypeptidase. Clearly, more experimentation is needed to clarify this hypothesis. Detailed studies elaborating on the metal specificity as well as true dose-responses are currently being pursued in our laboratory.

The increased expression of earthworm genes in response to heavy metals has been shown previously. For example, elevated mitochondrial expression is a reflection of the increase in energy demand, due to numerous triggers, including heavy metal detoxification (Stürzenbaum *et al.* 1998d). On the other hand, the expression of metallothionein is a far more metal specific response. Indeed, metallothionein transcripts can be elevated in excess of three orders of magnitude during exposure to cadmium, but only one order in response to copper (Stürzenbaum *et al.* 1998b). Whilst metallothionein and carboxypeptidase show similar expression characteristics in response to copper, it is worthwhile noting that, in contrast to metallothionein, carboxypeptidase seems to be unaffected by cadmium. The benefits of monitoring two (or more) functionally

distinct biomarkers have been highlighted by others (Petersen *et al.* 1997). This notion has general implications in the ecotoxicological applications of molecular genetic biomarkers, particularly in light of gene array technology and its potential use as a powerful tool in the assessment of soil quality and pollution impact. The immediate challenge in environmental diagnostics is to identify a suite of characterized genes that show differential responses. The majority may be non-specific indicators of stress that do not distinguish between chemical, physical or, indeed, biological stressors; some may be indicators of a broad class of loosely-related chemical stressors; others may be more-or-less toxicant specific.

Acknowledgements

This study was supported financially by the British Natural Environment Research Council (NERC grants GR9/02083 and GT5/94/ALS) and the Royal Society.

References

Auld DS. 1998 Carboxypeptidase A. In: Barrett AJ, Rawlings ND, Woessner JF. eds. *Handbook of proteolytic enzymes*. London, UK: Academic Press: pp. 1321–1326.

Barrett AJ, Rawlings ND, Woessner JF. 1998 *Handbook of Proteolytic Enzymes*, London, UK: Academic Press: pp. 1318–1351.

Campbell W, Okada H. 1989 An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits. *Biochem Biophys Res Commun* **162**, 933–939.

Chen Z, Mayer LM. 1998 Digestive proteases of the lugworm (*Arenicola marina*) inhibited by Cu from contaminated sediments. *Environ Toxicol Chem* **17**, 433–438.

Chikuma T, Matsumoto K, Furukawa A, Nakayama N, Yajima R, Kato T, Ishii Y, Tanaka A. 1996 A flurometric assay for measuring deamidase (lysosomal protective protein) using high-performance liquid chromatography. *Anal Biochem* **233**, 36–41.

Colombo S, Toietta G, Zecca L, Vanoni M, Tortora P. 1995 Molecular cloning, nucleotide sequence, and expression of a carboxypeptidase-encoding gene from the Achaebacterium *Sulfolobus solfataricus*. *J Bact* **177**, 5561–5566.

Folk JE, Piez KA, Carroll WR, Gladner JA. 1960 Carboxypeptidase B. Purification and characterization of the porcine enzyme. *J Biol Chem* **263**, 17828–17836.

Hendricks D, Scharpe S, van Sande M, Lommaert, MP. 1989 Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N *J Clin Chem Clin Biochem* **27**, 277–285.

Hofman T. 1985. Metalloproteinases. In: Harrison PM. ed. *Metalloproteinases Part 2: metals proteins with non-redox roles.* London, UK: Macmillan Press: pp. 1–64.

Itoh K, Takiyama N, Kase R, Kondoh K, Sano A, Oshima A, Sakuraba H, Suzuki Y. 1993 Purification and characterisation of human lysosomal protective protein expressed in stably transformation Chinese hamster ovary cells. *J Biol Chem* **268**, 1180–1186.

Laethem RM, Blumentopf TA, Cory M, Elwell L, Moxham CP, Ray PH, Walton LM, Smith GK. 1996 Expression and characterization of human pancreatic preprocarboxypeptidase A1 and preprocarboxypeptidase A2. *Arch Biochem Biophys* **322**, 8–18.

Morgan JE, Morgan AJ. 1990 The distribution of cadmium, copper, lead, zinc and cadmium in the tissues of the earthworm *Lumbricus rubellus* sampled from one uncontaminated and four polluted soils. *Oecol* **64**, 381–388.

OECD. 1984 Guideline for testing chemicals, No 207. Earthworm acute toxicity test. Adopted 4 April 1984, Organisation for Economic Cooperation and Development, Paris.

Petersen SN, Lundebye A-K, Depledge MH. 1997 Field application of metallothionein and stress protein biomarkers in the shore crab (*Carcinus maenas*) exposed to trace metals. *Aquatic Toxicol* **37**, 183–200.

Sarkar B. ed. 1999 *Metals and Genetics*, New York: Plenum.

Streit B. 1984 Effects of high copper concentrations on soil invertebrates (earthworms and oribatid mites). Experimental results and a model. *Oecol* **64**, 381–8.

Stürzenbaum SR, Kille P, Morgan AJ. 1998a Heavy metal induced molecular responses in the earthworm *Lumbricus rubellus*, genetic fingerprinting by directed differential display. *Appl Soil Ecol* **9**, 495–500.

Stürzenbaum SR, Kille P, Morgan AJ. 1998b The identification, cloning and characterization of earthworm metallothionein. *FEBS Lett* **431**, 437–442.

Stürzenbaum SR, Kille P, Morgan AJ. 1998c Identification of heavy metal induced changes in the expression patterns of the translationally controlled tumor protein (TCTP) in the earthworm *Lumbricus rubellus*. *Biochem et Biophys Acta* **1398**, 294–304.

Stürzenbaum SR, Kille P, Morgan AJ. 1998d. Identification of new heavy-metal-responsive biomarkers in the earthworm. In: Sheppard S, Bembridge J, Holmstrup M, Posthuma L. eds. *Advances in earthworm ecotoxicology*, Pensacola, FL, USA: Society of Environmental Toxicology and Chemistry (SETAC): pp. 215–224.

Tranchemontage J, Michaud L, Potier M. 1990 Deficient lysosomal carboxypeptidase activity in galactosialidosis. *Biochem Biophys Res Commun* **168**, 22–29.