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# Distribution of manganese in rat pancreas and identification of its primary binding protein as pro-carboxypeptidase B

Hiroshi KODAMA,*†‡ Nobuhiro SHIMOJO† and Kazuo T. SUZUKI*§
*National Institute for Environmental Studies, Onogawa, Tsukuba, Ibaraki 305,
and †Institute for Environmental Sciences, University of Tsukuba, Tennodai, Tsukuba, Ibaraki 305, Japan

Distribution of manganese (Mn) and its binding to specific proteins were examined in rat pancreas. A $MnCl_2$ solution was injected subcutaneously into Wistar rats daily at a single dose of 15 mg of Mn/kg body weight for 10 days and the animals were killed 1 day after the last injection. The concentration of Mn in the pancreas increased considerably from $1.4 \pm 0.2$ (control) to $13.3 \pm 3.7$ $\mu$g/g wet tissue by the repeated injection of Mn. The distribution of Mn in the soluble fraction of the pancreas (170000 *g* supernatant) was determined on a gel-filtration column (Asahipak GST-520) using an h.p.l.c.–inductively coupled argon plasma atomic-emission spectrometry (i.c.p.) technique. The metal was eluted as a single peak in the high-molecular-mass protein fraction, where Mn had been observed as a small peak in the control profile, suggesting that the administered Mn was bound to the same Mn-binding component as that in the control. On the basis of enzymic and chemical characterization of the protein, it was identified as a zymogen of carboxypeptidase B (pro-carboxypeptidase B, pro-CPB). The elution profiles of the protein by h.p.l.c.–i.c.p. indicated that Mn and zinc (Zn) were bound to the zymogen with a molar ratio of 1:4 in normal rat pancreas. Mn bound to the zymogen was easily replaced by Zn *in vitro*, suggesting that Mn was bound to the Zn-binding site and that the binding affinity to Zn was higher than that to Mn. The present results indicate that pro-CPB is the primary Mn-binding protein in the pancreas of control and also Mn-administered rats.

## INTRODUCTION

In mammalian tissues, pancreas contains a relatively high level of manganese (Mn). Mn is believed to be an essential trace element for pancreatic exocrine and endocrine functions, since Mn deficiency impairs these functions, including an elevation of tissue amylase content [1,2] and an abnormal insulin secretion that results in glucose intolerance [3,4]. In addition, Mn is known to stimulate protein synthesis [5,6] and to regulate amylase release [7] in isolated pancreatic acini. On the other hand, chronic Mn exposure has been reported to cause physiological [4,8] and histological [9] abnormalities in the pancreas of experimental animals, which appears to be attributable to a significant accumulation of Mn in this organ [10–14]. Sakurai *et al.* [13] demonstrated that, when rats were injected with $MnCl_2,4H_2O$ at a dose of 15 mg/kg body weight once a day for 2 weeks, the Mn content in the pancreas was 2–40 times higher than those in other tissues. The pancreas therefore appears to be a specific target organ for the accumulation and toxicity of Mn.

It has thus been recognized that Mn is selectively taken up by the pancreas and the metal is associated with pancreatic functions, although the metabolic fate of Mn in this organ is not well known. Mn taken up by the pancreas has been thought to bind initially to intracellular proteins of high affinity for Mn (Mn-binding proteins) and thereafter to be delivered to metalloenzymes and metalloproteins which require the metal as a constituent, for example Mn-superoxide dismutase, or to be excreted from the organ in the pancreatic juice [14,15]. However, Mn-binding proteins have not been previously detected or identified in the pancreas.

The present paper constitutes, as far as we know, the first report concerning the chromatographic distribution of Mn in the pancreas before and after parenteral Mn administration. Mn-binding proteins were analysed on both gel-filtration and anion-exchange columns using the h.p.l.c.–inductively coupled argon plasma atomic-emission spectroscopy (i.c.p.) technique [16–20] developed recently in our laboratory. These columns were eluted under neutral-buffer conditions (pH 7.4) to avoid the dissociation of the metal from the proteins.

## MATERIALS AND METHODS

### Reagents

Bovine chymotrypsin, hippuryl-L-arginine, carbobenzoxy-glycyl-L-phenylalanine and BSA (fraction V powder) were from Sigma (St. Louis, MO, U.S.A.); marker proteins for electrophoresis were from Bio-Rad (Richmond, VA, U.S.A.).

### Mn administration

**Animals.** Male Wistar rats (6 weeks old; JCL; Clea Japan, Tokyo, Japan) were injected subcutaneously with $MnCl_2$ at a dose of 15 mg Mn/kg body weight for 10 consecutive days and killed 1 day after the last injection by exsanguination under pentobarbital anaesthesia. Untreated animals served as controls. Pancreas, liver and kidneys were removed from control and Mn-administered rats and stored at −80 °C until analysis.

**Determination of Mn concentration in tissues.** A 0.2 g portion of tissue was wet-digested with 1.0 ml of mixed acids [$HNO_3/HClO_4$, 5:1 (v/v)] at 100–120 °C. The solution was concentrated to about 0.1 ml and diluted to 5.0 ml with double-distilled water. Concentrations of Mn and other elements were determined simultaneously by i.c.p. (model JY48PVH instrument; Seiko Instruments and Electronics Ltd., Tokyo, Japan). The operating conditions for i.c.p. were same as those reported previously [16].

Abbreviations used: i.c.p., inductively coupled argon plasma atomic-emission spectrometry; (pro-)CPB, (pro-)carboxypeptidase B; a.a.s., atomic-absorption spectrometry; CPA, carboxypeptidase A; $R_t$, retention time.

‡ Present address: Basic Research Group, Tsukuba Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., 5-2-3 Tohkodai, Tsukuba, Ibaraki 300-26, Japan.

§ To whom correspondence should be sent.

Statistical analysis was performed by Student's $t$ test. A $P$ value of less than 0.05 was considered to be significant.

**H.p.l.c.–i.c.p. determination of distribution profiles.** Pooled pancreas samples from control or Mn-administered rats were homogenized in three volumes of 0.1 M-Tris/HCl buffer solution, pH 7.4, containing 0.25 M-glucose, using a glass–Teflon homogenizer under ice/water cooling. The homogenates were centrifuged at 170000 $g$ for 70 min at 4 °C. The resulting supernatants were designated as the crude extracts. All extracts were sealed in an atmosphere of $N_2$ gas and stored at −80 °C until analysis.

A 0.1 ml portion of the crude extract was applied on a gel-filtration column (Asahipak GST-520 column, 7.5 mm internal diameter × 500 mm; Asahi Chemical Industries, Kawasaki, Japan) [26]. The column was eluted with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.9% NaCl and 0.05% $NaN_3$ at a flow rate of 1.0 ml/min on an h.p.l.c. apparatus (HPLC Series Instrument 340; Beckman, Berkeley, CA, U.S.A.). The elution buffer was degassed by connecting a degasser (Shodex Degas; Showa Denko, Tokyo, Japan) just before the h.p.l.c. pump. Absorbances at 254 and 280 nm were monitored continuously with a dual-wavelength u.v. detector (model 152, Altex, Berkeley, CA, U.S.A.). The eluate from a GST column was introduced into the nebulizer of an i.c.p. instrument, and concentrations of Mn, sulphur (S), Zn, copper (Cu), and iron (Fe) were determined simultaneously and continuously by the h.p.l.c.–i.c.p. method [16].

### Mn-binding protein

**Electrophoretic analysis.** SDS/PAGE was carried out as described by Laemmli [21], with a separating gel of 12.5% (w/v) polyacrylamide. After electrophoresis, gels were stained with 0.025% (w/v) Coomassie Brilliant Blue R-250 in propan-2-ol/acetic acid/water (5:2:13, by vol.) and then were destained by diffusion in 10% (v/v) acetic acid.

**H.p.l.c. separation.** A Mn-binding protein in normal rat pancreas was purified on an anion-exchange column after preliminary separation on a gel-filtration column using the following procedure.

A 3.0 ml portion of the crude extract (supernatant) prepared from normal rat pancreas (male Wistar rats, 14 weeks old; JCL, Clea Japan) was applied on a gel-filtration column (Asahipak GS-520P column, 21 mm internal diameter × 500 mm; Asahi Chemical Industries). The column was eluted with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.1% $NaN_3$, degassed before use, at a flow rate of 5.0 ml/min on an h.p.l.c. instrument and successive 2.5 ml fractions of the eluate were collected. The concentration of Mn in the obtained fraction was determined by atomic-absorption spectrometry (a.a.s.; model 170-50A instrument; Hitachi, Tokyo, Japan). The Mn fraction between $R_t$ 17.5 and 19.5 min was pooled as the 'GS fraction'.

A 6.0 ml portion of the GS fraction was applied on an anion-exchange column (TSKgel DEAE-3SW column; Tosoh, Tokyo, Japan). The column was eluted with a linear concentration gradient of Tris/HCl buffer solution, pH 7.4, from 10 to 400 mM, at a flow rate of 1.0 ml/min, and 1.0 ml fractions were collected. The Mn fraction between $R_t$ 31.0 and 33.0 min was designated the 'DEAE fraction', and a 1.0 ml portion of the GS fraction was applied on a DEAE column and eluted under the same conditions. The elution profiles of elements from the DEAE column were determined by the h.p.l.c.–i.c.p. method.

**Amino-acid-sequence analysis.** In order to determine a part of the amino acid sequence, the Mn-binding protein was further purified on a reversed-phase h.p.l.c. column after digestion with chymotrypsin.

The Mn-binding protein purified on a DEAE column was incubated with bovine chymotrypsin at a concentration of 50 $\mu$g/ml for 1 h at 37 °C. A 6.0 ml portion of the digest (containing 50 $\mu$g of Mn-binding protein/ml) was applied on a reversed-phase h.p.l.c. column (TSKgel TMS-250 column, 4.6 mm internal diameter × 75 mm; Tosoh). The TMS-250 column was eluted with a linear concentration gradient of acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 1.0 ml/min. Absorbances at 254 and 280 nm were monitored. Eluates corresponding to peaks of high absorbance were collected and freeze-dried under vacuum.

Sequence analysis of a chymotryptic digest of the Mn-binding protein was done with a gas-phase sequencer (model 470A; Applied Biosystems, Foster City, CA, U.S.A.) and an on-line-connected h.p.l.c. system (model 120A; Applied Biosystems).

**Assay of carboxypeptidase activities.** Assay for carboxypeptidase A and B (CPA and CPB) activities requires prior activation of the zymogens. Samples were incubated with bovine chymotrypsin (10 $\mu$g/ml) for 30 min at 37 °C and peptidase activities were then determined.

CPB activity was determined spectrophotometrically at 25 °C by Folk's method [22]. After incubation of the Mn-binding protein with 10 $\mu$g of bovine chymotrypsin/ml for 1 h at 37 °C, its peptidase activity was measured in 36 mM-Tris/HCl buffer, pH 8.0, containing 1.5 mM-*N*-hippuryl-L-arginine. A unit of activity was defined as the amount of enzyme that catalysed the hydrolysis of substrate at the rate of 1 $\mu$mol/min under the conditions employed.

CPA activity was determined by the rate of hydrolysis of carbobenzoxyglycyl-L-phenylalanine as described by Petra [23].

Protein concentration was determined by the Lowry method [24], with BSA as the standard.

## RESULTS

### Effect of Mn administration on the Mn concentration in organs

Concentrations of Mn in the pancreas, liver and kidney of control and Mn-administered rats were determined by i.c.p. as shown in Table 1. Mn administration resulted in a significant increase in the Mn level of all tissues compared with the respective controls, the highest level being observed in pancreas. The Mn distribution among cytosolic molecular components was therefore studied in pancreas by using the h.p.l.c.–i.c.p. technique.

### Distribution of Mn in the supernatant fraction of pancreas on a gel-filtration h.p.l.c. column

Fig. 1 demonstrates the elution profiles of several elements on a gel-filtration column (GST-520 column) in the crude pancreas extracts prepared from control and Mn-administered rats by the h.p.l.c.–i.c.p. method. The concentration of Mn in the crude pancreas extract increased markedly from 0.15 (control) to 0.68 $\mu$g/ml (Mn-administered).

**Table 1. Concentration of Mn in tissues of control and Mn-treated rats**

Values are means ± S.D. for six rats. * Indicates significantly different from the respective control, $P < 0.05$.

| | [Mn] ($\mu$g/g of wet tissue) | |
|---|---|---|
| Organ | Control | Mn-treated |
| Pancreas | 1.4 ± 0.2 | 13.3 ± 3.7* |
| Liver | 2.4 ± 0.1 | 4.6 ± 0.5* |
| Kidney | 0.9 ± 0.1 | 7.7 ± 0.7* |

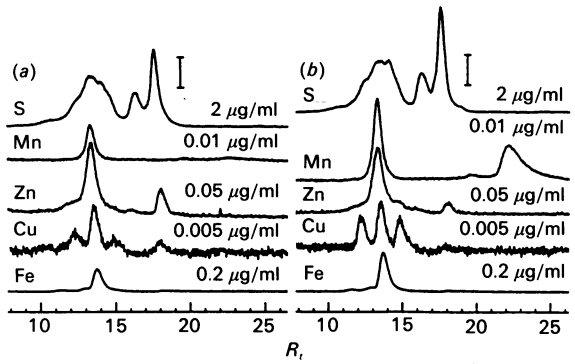


**Fig. 1. Distributions of several elements in the pancreas supernatant prepared from before (*a*) or after (*b*) Mn administration**

Male Wistar rats were injected subcutaneously with $MnCl_2$ at a dose of 15 mg of Mn/kg body weight for 10 consecutive days and killed 1 day after the last injection. Animals without any treatment served as controls. A 0.1 ml portion of the pancreas supernatant prepared from the control (*a*) or Mn-administered (*b*) rats was applied on an Asahipak GST-520 column. The column was eluted with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.9% NaCl and 0.05% $NaN_3$ at a flow rate of 1.0 ml/min. Concentrations of elements (S, Mn, Zn, Cu and Fe) in the eluate were determined by i.c.p. The vertical bar indicates the detection level of the respective elements at the concentrations shown.

In the control profile, Mn was eluted as a single peak at a retention time ($R_t$) of 13.2 min (Fig. 1*a*), and its peak intensity increased considerably after the administration of Mn (Fig. 1*b*). The major Zn peak was present with the same $R_t$ as that of Mn (13.2 min). In the Mn profile of Fig. 1(*b*), a broad Mn peak was observed in the low-molecular-mass protein fraction at $R_t$ 22.0 min. The broad peak at $R_t$ 22.0 min was tentatively assigned as ionic Mn, since Mn salt ($MnCl_2$) examined under the present conditions was eluted at the same retention time ($R_t$ 22.0 min; results not shown).

In order to determine whether Mn and Zn were bound to the same component, distributions of the two metals on a GST-520 column were examined *in vitro* after incubating the crude pancreas extract prepared from control rats with Zn or Mn salt. When Zn was incubated with the extract, the Mn peak at $R_t$ 13.2 min decreased in intensity with an increase of Zn intensity (Fig. 2*b*). On the other hand, incubation with Mn caused an increase of the Mn peak intensity without affecting the distribution of Zn (Fig. 2*c*). This result indicates that Mn may be bound to the Zn-binding site of the component, and that its binding affinity for Zn is higher than that for Mn.

Distributions of Mn were also examined in the supernatant prepared from rat liver and kidney under the same conditions. No Mn peak was observed at $R_t$ 13.2 min in these two organs (results not shown).

### H.p.l.c. separation

The Mn- and/or Zn-binding protein in normal rat pancreas detected at $R_t$ 13.2 min on a GST-520 column was further analysed on an anion-exchange column (DEAE column), as shown in Fig. 3. Zn was separated into two major peaks, whereas Mn was eluted as a single peak at the same retention time ($R_t$ 31.4 min) as that of the slower Zn peak in normal rats. The minor Fe peak was also present at the same retention time as that of Mn.

After preparation on a DEAE column, the Mn-binding protein was subjected to SDS/PAGE and the protein was detected as a single protein, as shown in Fig. 4, lane 2. Its $M_r$ was estimated to be about 45000. The elution profile on the DEAE column indicated that Mn and Zn were bound to the protein at a molar ratio of 1:4 in normal rats (Fig. 3).

Results of purification of the Mn-binding protein in normal rats pancreas are summarized in Table 2. The amounts of metal bound to the Mn-binding protein were calculated to be 0.06 and 0.23 atoms of Mn and Zn per molecule respectively.

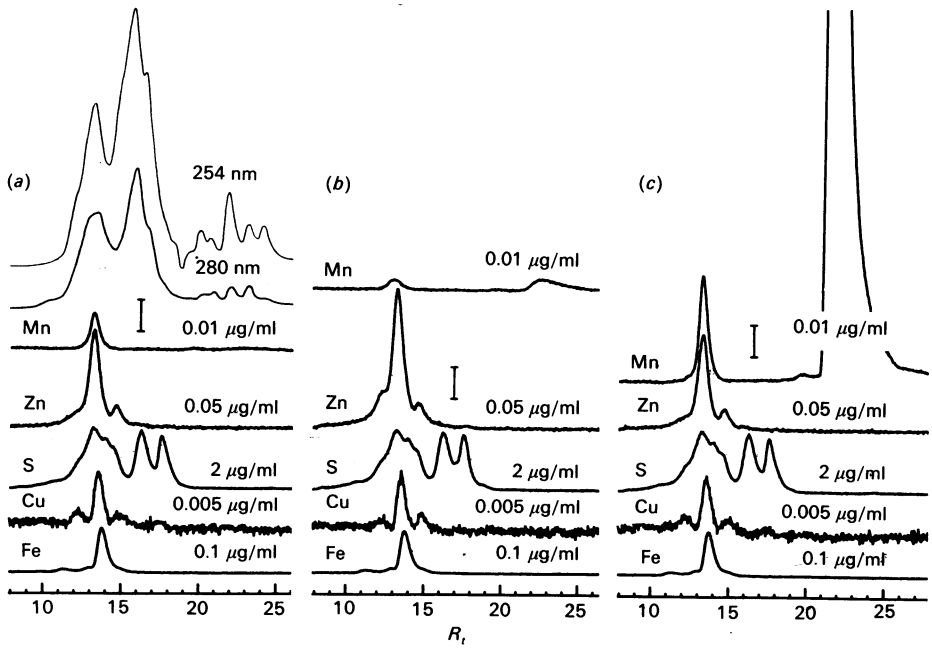


**Fig. 2. Changes in the distributions of elements in the pancreas supernatant on a gel-filtration column after incubation with Mn or Zn salt**

The pancreas supernatant prepared from normal rats was mixed with saline (*a*), $ZnCl_2$ (*b*) or $MnCl_2$ (*c*) at a concentration of 10 μg of Zn or Mn/ml and incubated for 10 min at 37 °C. The distribution profiles of elements in the samples were determined by h.p.l.c.–i.c.p.

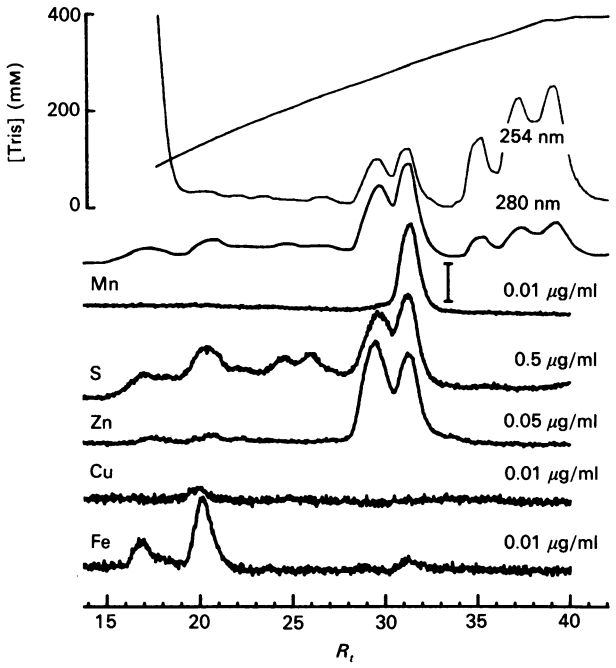


**Fig. 3. Distribution profiles of several elements on an anion-exchange column for the Mn-containing fraction separated on a GS column**

Pancreases of normal rats (male Wistar strain, 14 weeks old) were used. A 6.0 ml portion of the Mn-containing fraction separated on an Asahipak GS-520P column was applied on a TSKgel DEAE-3SW column. The column was eluted with a linear concentration gradient of Tris/HCl buffer solution from 10 to 400 mM (diagonal line) at a flow rate of 1.0 ml/min. The concentrations of elements in the eluate were determined simultaneously by i.c.p. Absorbances at 254 and 280 nm were monitored.

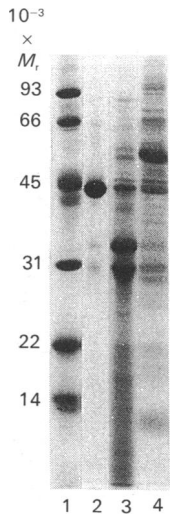


**Fig. 4. SDS/PAGE of the Mn-binding protein**

The Mn-containing fractions separated on Asahipak GS-520 and TSKgel DEAE-3SW columns were subjected to SDS/PAGE [12% (w/v) polyacrylamide]. Lane 1, marker proteins: 93, phosphorylase *b* ($M_r$ 92500); 66, serum albumin (66200); 45, ovalbumin (45000); 31, carbonic anhydrase (31000); 22, soybean trypsin inhibitor (21500); 14, lysozyme (14400). Lane 2, eluate from a TSKgel DEAE-3SW column. Lane 3, eluate from an Asahipak GS-520P column. Lane 4, crude extract (supernatant) of normal rat pancreas. After electrophoresis, sample proteins adsorbed on the gel were stained with 0.025% Coomassie Brilliant Blue.

**Table 2. Purification of Mn-binding protein from normal rat pancreas**

| Step | Metal content (ng/mg of protein) Mn | Zn |
|---|---|---|
| (*a*) Supernatant | 5 | 100 |
| (*b*) Eluate from a GS-520P column | 16 | 160 |
| (*c*) Eluate from a DEAE-3SW column | 68 | 330 |

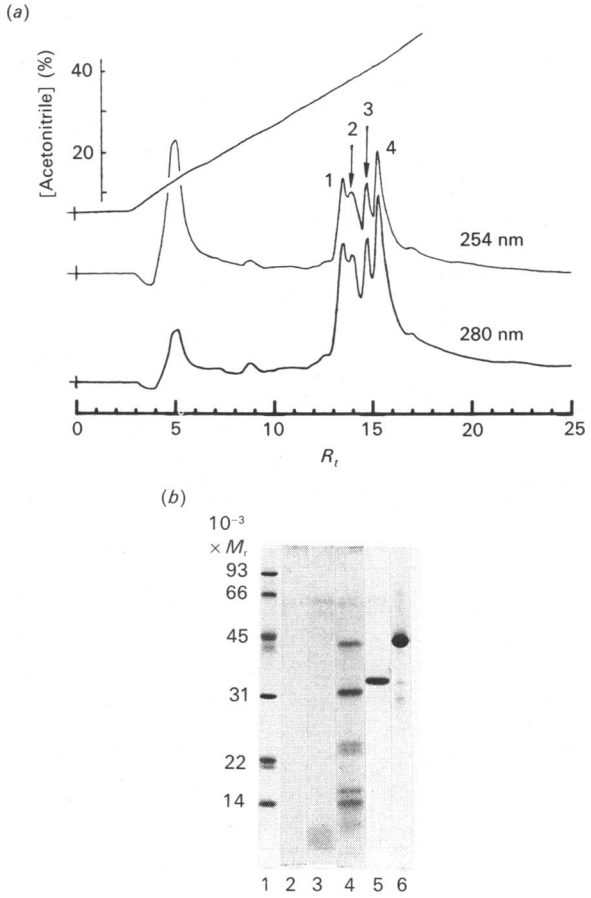


**Fig. 5. A reversed-phase h.p.l.c separation of the chymotryptic digests of the Mn-binding protein**

(*a*) The Mn-binding protein isolated on a TSKgel DEAE-3SW column was incubated with chymotrypsin (50 μg/ml) for 1 h at 37 °C. A 6.0 ml portion of the digest was applied on a TSKgel TMS-280 column and the column was eluted with a linear concentration gradient (diagonal line) of acetonitrile (0.05% trifluoroacetic acid) at a flow rate of 1.0 ml/min. Absorbances at 254 and 280 nm were monitored. (*b*) Fractions on a TMS-250 column corresponding to large absorbance peaks were collected and subjected to electrophoresis as described in the legend to Fig. 4. Lane 1, molecular marker proteins. Lanes 2–5, fractions on a TMS-250 column [corresponding to 1–4 in (*a*) respectively]. Lane 6, eluate from a TSKgel DEAE-3SW column.

## Determination of amino acid sequence

Fig. 5(*a*) shows the elution profile on a reversed-phase column (TMS-250 column) for the chymotryptic digests of the Mn-binding protein. The amino acid sequence of a digest of $M_r$ 35000 (Fig. 5*a*, peak 4; Fig 5*b*, lane 5) was determined (Table 3*a*). The

**Table 3. Comparison of the partial amino acid sequence of the Mn-binding protein (*a*; Mn-BP) with that of rat CPB (*b*)**

The amino acid sequence of a fragment of the Mn-binding protein determined in the present study was compared with that of rat CPB [24]. Xaa, not detectable.

| Cycle no.... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 24 | 25 | 26 | 27 | 28 | 29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (*a*) Mn-BP | | | | | | | | | | Ala- | Ser- | Gly- | His- | Ser- | Tyr- | Thr- | Lys- | Xaa- | Asn- | Lys- | Trp- | Glu- | Thr- | Ile- | Glu- | Ala- | Trp- | Ile- | Gln- | Gln- | Val |
| (*b*) Rat CPB | Ser- | His- | Thr- | Arg- | Ala- | Ser- | Gly- | His- | Ser- | Tyr- | Thr- | Lys- | Tyr- | Asn- | Lys- | Trp- | Glu- | Thr- | Ile- | Glu- | Ala- | Trp- | Ile- | Gln- | Gln- | Val- | Ala- | Thr- | | | |

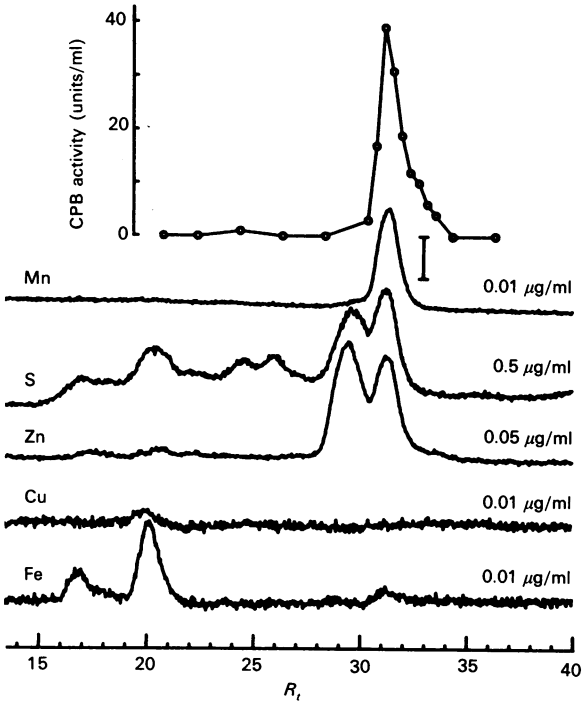


**Fig. 6. Elution profiles, on an anion-exchange column, of CPB activity and Mn**

Anion-exchange h.p.l.c. was performed under the same conditions as those described in the legend to Fig. 3. Each 0.4 ml eluate from a TSKgel DEAE-3SW column was collected and incubated with chymotrypsin (10 μg/ml) for 30 min at 37 °C. After the incubation, CPB activity in the samples was determined spectrophotometrically by the method of Folk [22]. A unit of activity corresponds to the hydrolysis of 1 μmol of *N*-hippuryl-L-arginine/min. The Mn distribution in the eluate was determined by i.c.p.

amino acid sequence of the digest of the Mn-binding protein was same as that of rat CPB (Table 3*b*) reported by Clauser *et al.* [25].

### CPB activity

After limited proteolysis with chymotrypsin, the Mn-binding protein purified from normal rat pancreas demonstrated a CPB specific activity of 185 units/mg of protein.

Fig. 6 demonstrates the elution profiles of CPB activity and of the metals of the GS fraction on a DEAE column. The peak of CPB activity was present at the same retention time as that of the Mn peak. The CPA activity was observed at the same time as the 'faster' peak of Zn (results not shown).

## DISCUSSION

Previous reports have demonstrated that chronic Mn administration to experimental animals resulted in a significant accumulation of Mn in the pancreas [11,13]. However, its intracellular ligands have not been previously reported. The present study has demonstrated, for the first time as far as we know, the distribution of Mn in crude extracts of rat pancreas. The elution profiles of various metals on an Asahipak GST-520 column revealed that Mn was mostly bound to a single protein having a higher affinity for Zn in normal rats. The Mn-binding protein was identified as pro-CPB on the basis of the amino-acid-sequence identity, physicochemical and enzymological properties. The $M_r$ (45000) corresponded well to those of human (47000), porcine (48900) and lungfish (45000) pro-CPB reported previously [27–29].

The GST-520 column was prepared in our laboratory from its original column, Asahipack GS-520, by methylation of the residual carboxy groups, in order to minimize the interaction of metals with ligands present in substances and in gel materials [26]. We have previously demonstrated that the column is more efficient at detecting serum Zn-binding proteins such as globulins and albumin [26]. In addition, when a Mn salt ($MnCl_2$) was applied and eluted under the present conditions, it was detected as a relatively sharp peak on the column ($R_t$ 22.0 min; results not shown), indicating that the interaction between Mn and column materials is minimal. The GST-520 column is therefore suitable for detecting and separating Mn- and Zn-binding proteins in biological samples without dissociating the metals from their ligands.

With regard to metal contents, CPB prepared from acetone-dried powders of porcine pancreas was shown to contain 1 g-atom of Zn per molecule of $M_r$ 34000 [30]. Bovine pro-CPA-S6 was reported to contain $0.84 \pm 0.16$ g-atom/molecule ($M_r$ 87000) and significant amounts of iron and nickel [30]. However, as far as we know, there are no precise data concerning the metal contents of pro-CPB. In the present study, pro-CPB purified from normal rat pancreas was calculated to contain 0.06 g-atom of Mn and 0.23 g-atom of Zn per molecule of $M_r$ 45000 (Table 2). The h.p.l.c.–i.c.p. profiles on a DEAE-3SW column also showed that Mn and Zn were bound to the zymogen at a molar ratio of 1:4 (Fig. 3). Furthermore, an addition of Mn to the pancreatic extract *in vitro* induced an increase of the Mn peak intensity corresponding to pro-CPB (Fig. 2*c*), suggesting that the zymogen is present to some extent as the apo-form.

The specific contents of Mn and Zn during the purification procedures are listed in Table 1. The specific content of Mn (ng of Mn/mg of protein) was increased 3.2-fold (16/5) by the gel-filtration procedure, whereas that of Zn was increased only 1.6-fold (160/100). This difference is probably due to the absence of other Mn-binding proteins or the presence of other Zn-binding proteins, as can be seen in the distribution profiles of Mn and Zn in Fig. 1. Furthermore, the specific content of Mn was increased 4.25-fold (68/16) by the ion-exchange procedure, whereas that of Zn was increased 2.06-fold (330/160). This 2-fold difference is due to the removal of pro-CPA that binds Zn, but not Mn, during the procedure. Thus the Mn/Zn ratio was not very much decreased in any of the steps, suggesting that even Mn with weaker affinity to pro-CPB was not lost during the purification procedures.

The concentration of Mn was increased 9.5-fold (13.3/1.4) in the whole pancreas by exposure to Mn, but only 4.5-fold (0.68/0.15) in the supernatant. The result indicates that Mn was distributed such that there was more in the non-soluble fraction. Pro-CPB is known to be present in lysosomes of acinal cells of pancreas and, in fact, the present Mn-binding protein (pro-CPB) was extracted from the tissue in larger quantity after prolonged homogenization (results not shown), indicating that the recovery of Mn in the soluble fraction depends on the conditions of homogenization. Furthermore, recent electron-microscopic work on the subcellular distribution of Mn revealed that, when Wistar rats were administered with $MnCl_2,4H_2O$ at a dose of 5 mg/kg body weight twice a day for 3 weeks, Mn was observed to be concentrated into lysosome-like granules in the pancreas (M. Nishida, H. Sakurai & H. Ishizuka, unpublished work), a finding that accords with the present results.

The present data indicate that exogenously administered Mn is bound to the pre-existing pro-CPB in the rat pancreas (Fig. 1). The Zn peak intensity of pro-CPB was hardly changed by exposure to Mn, suggesting that Mn taken up by the pancreas is bound to the apo form of the pre-existing pro-CPB. Thus pro-CPB is the primary Mn-binding protein in the pancreas of both normal and Mn-administered rats, implying that the zymogen may have roles in metabolic behaviour of Mn in the pancreas.

We have examined the distribution of Mn not only in the pancreas, but also in the liver and kidneys (results not shown). Injections of Mn increased the distributions of the metal in all tissues. Furthermore, the increased Mn in the soluble fractions was distributed only to the pre-existing Mn-binding proteins in the three tissues. In other words, Mn binds only to the pre-existing Mn-binding proteins, and no new Mn-binding proteins were induced or detected. It remains unclear why Mn binds only the pre-existing Mn-binding proteins and not to general proteins as usually observed for Zn, Cd and other metals. However, this characteristic property of Mn may explain the rapid clearance of the metal in the body, as reported previously [31].

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