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The insulin-secretory-granule carboxypeptidase H

Purification and demonstration of involvement in proinsulin processing

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A carboxypeptidase B-like enzyme was detected in the soluble fraction of purified insulin secretory granules, and implicated in insulin biosynthesis. To investigate the role of this activity further, we purified the enzyme from rat insulinoma tissue by gel-filtration chromatography and affinity elution from *p*-aminobenzoyl-arginine. A yield of 42%, with a purification factor of 674 over the homogenate, was achieved. Analysis of the purified carboxypeptidase by SDS/polyacrylamide-gel electrophoresis under either reducing or non-reducing conditions showed it to be a monomeric protein of apparent M_r 55000. The preparation was also homogeneous by high-performance gel-filtration chromatography. The enzyme bound to concanavalin A, showing it to be a glycoprotein. Amino acid analysis or chemical deglycosylation and SDS/polyacrylamide-gel electrophoresis indicated a protein M_r of 50000, suggesting a carbohydrate content of approx. 9% by weight. The purified enzyme was able to remove basic amino acids from the *C*-terminus of proinsulin tryptic peptides to generate insulin, but did not further degrade the mature hormone. It was inhibited by EDTA, 1,10-phenanthroline and guanidinoethylmercaptosuccinic acid, and stimulated 5-fold by $CoCl_2$. The pH optimum of the conversion of diarginyl-insulin into insulin was in the range 5–6, with little activity above pH 6.5. Activity was also expressed towards a dansylated tripeptide substrate (dansyl-phenylalanyl-leucyl-arginine; K_m = 17.5 μM), and had a pH optimum of 5.5. These properties are indistinguishable from those of the activity located in secretory granules, and are compatible with the intragranular environment. The insulin-secretory-granule carboxypeptidase shared several properties of carboxypeptidase H from bovine adrenal medulla and pituitary. We propose that the carboxypeptidase that we purified is the pancreatic isoenzyme of carboxypeptidase H (crino carboxypeptidase B; EC 3.4.17.10), and is involved in the biosynthesis of insulin in the pancreatic β-cell.

INTRODUCTION

Insulin, in common with most polypeptide hormones and many bioactive peptides, is synthesized as a precursor, proinsulin, from which the active protein is excised by limited proteolysis at sites marked by pairs of basic amino acids (for reviews see Docherty & Steiner, 1982; Loh *et al.*, 1984).

Consideration of the sites of cleavage within proinsulin, and of the structure of the endogenous intermediates isolated from bovine pancreas, led to the suggestion that enzymes of 'trypsin-like' and 'carboxypeptidase B-like' specificity could be responsible for the conversion of proinsulin into insulin *in vivo* (Nolan *et al.*, 1971). This hypothesis was supported by the demonstration of a 'carboxypeptidase B-like' activity in crude secretory-granule fractions prepared from rat islets of Langerhans with, as substrate, biosynthetic [*Arg*-^{3}H]proinsulin which had been cleaved at the pairs of basic residues by limited trypsinolysis (Kemmler *et al.*, 1973). Like the exocrine pancreatic carboxypeptidase B, this enzyme was inhibited by metal-ion chelators, but it could be distinguished by its acidic rather than alkaline pH optimum (Zuhlke *et al.*, 1977).

An enzyme with similar properties has also been identified in purified insulin-secretory granules prepared from a transplantable rat insulinoma (Docherty & Hutton, 1983). The tumour, originally induced by X-ray irradiation, consists predominantly of well-granulated β-cells, and has a high (pro)insulin content (Chick *et al.*, 1977). It converts proinsulin into insulin with kinetics indistinguishable from those observed in islets of Langerhans (Gold *et al.*, 1984).

The aim of the present study was to isolate, and further to characterize, the insulin-secretory-granule carboxypeptidase involved in proinsulin processing, by using the insulinoma as a source of material.

MATERIALS AND METHODS

Tissue

Pancreatic β-cell tumours were propagated in New England Deaconess Hospital (NEDH) rats fed *ad libitum*, as described previously (Hutton *et al.*, 1981). The fibrous capsule of each tumour was removed, and the tissue chopped into 1–2 mm fragments and rinsed in ice-cold buffered saline (Hanks & Wallace, 1949). All subsequent steps with the tissue were performed at 4 °C unless otherwise specified.

Purification of granule carboxypeptidase

Tissue (3–10 g wet wt.) from three to six animals was resuspended in 4 vol. of 25 mM-Tris/HCl, pH 8, containing 1 mM-phenylmethanesulphonyl fluoride

Abbreviations used: Dns-FLR, dansyl-phenylalanyl-leucyl-arginine; GEMSA, guanidinoethylmercaptosuccinic acid.

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(Sigma Chemical Co., Poole, Dorset, U.K.), homogenized in a glass tube homogenizer (Jencons Scientific, Leighton Buzzard, Beds., U.K.) with six strokes of a Teflon pestle driven at 600 rev./min, and further disrupted with two 15 s bursts of sonication at maximum power (MSE Soniprep 150, medium probe). The homogenate was centrifuged for 45 min at 37000 *g* ($r_{av.}$ = 8.3 cm; Sorvall SS34 rotor), and the supernatant retained. The pellet was twice re-extracted and centrifuged as described above.

The combined supernatants were adjusted to pH 6.5 by dropwise addition of 1 M-acetic acid, left for 15 min, and centrifuged for 30 min at 37000 *g*. The pellet was re-extracted in 4 vol. of 25 mM-Tris/acetate, pH 6.5, and centrifuged as before. The combined supernatants were made 50% saturated with $(NH_4)_2SO_4$ by the gradual addition of solid (BDH, Poole, Dorset, U.K.). After 30 min, the precipitated protein was collected by centrifugation for 20 min at 13200 *g* (SS34 rotor), and resuspended in approx. 2 vol. of 0.1 M-sodium acetate adjusted to pH 6 with HCl and containing 200 mM-NaCl (buffer A).

After dialysis for 16 h against 100 vol. of buffer A, insoluble material was removed by centrifugation for 30 min at 37000 *g*, and the supernatant was applied to a 2.6 cm × 95 cm (505 ml) column of AcA44 gel-filtration resin (LKB, S. Croydon, Surrey, U.K.) in buffer A at a flow rate of 20 ml/h. Fractions (4.6 ml) containing the peak of carboxypeptidase activity were pooled, and applied to a 0.8 cm × 2 cm (1 ml) affinity column of *p*-aminobenzoylarginine coupled to Sepharose 4B (215 μmol/ml of hydrated gel; gift from Dr. T. H. Plummer, New York State Department of Health, Albany, NY, U.S.A.) in buffer A at a flow rate of 24 ml/h. The column was washed with 20 ml of buffer A, followed by 50 ml of buffer A containing 1 M-NaCl, and then eluted with buffer A containing 1 M-arginine (BDH; chromatographically homogeneous). The column was first saturated with eluent, and the flow was arrested for 30 min and then restored. The pooled eluate was dialysed against 100 vol. of buffer A with three changes over 24 h, and finally stored at −20 °C.

Assay procedures

Protein was determined by the dye-binding method of Bradford (1976), with bovine serum albumin (Boehringer–Mannheim, Lewes, Sussex, U.K.) as standard.

Carboxypeptidase activity was determined by a modification of the method of Fricker & Snyder (1983). Samples in glass test tubes (10 mm × 63 mm) were preincubated for 5 min at 37 °C in 50 mM-sodium acetate adjusted to pH 5.5 with HCl and containing 0.1% Brij 35 (Sigma) with or without 12.5 μM-guanidinomercaptosuccinic acid (GEMSA; Cambridge Biosciences, Cambridge, U.K.) in a final volume of 400 μl. The reaction was initiated by the addition of 100 μl of a pre-warmed solution containing 50 mM-sodium acetate, pH 5.5, 0.1% Brij 35, 5 mM-$CoCl_2$ (BDH), and the substrate, 0.5 mM-dansylphenylalanyl-leucyl-arginine (Dns-FLR; Cambridge Biosciences), and terminated after 10 min by the addition of 50 μl of 1 M-HCl. The products were extracted into 1.5 ml of chloroform (BDH), and fluorescence in the organic phase was measured (excitation 360 nm, emission 510 nm; Perkin–Elmer LS5 instrument). Standardization was performed with Dns-phenylalanine (Sigma). Kinetic constants were determined by standard procedures as described by Cornish-Bowden (1981).

SDS/polyacrylamide-gel electrophoresis

Slab gels (12 cm × 15 cm × 0.15 cm) polymerized from 125 g of acrylamide/l and 1 g of *NN'*-methylenebisacrylamide/l (Electran grade; BDH) were prepared by a modification of the method of Laemmli (1970). Briefly, this involved the inclusion of 140 g of sucrose (BDH)/l and 10 mM-EDTA into the upper ('stacking') gel, and the use of tank buffers containing 50 mM-Tris base (Sigma), 384 mM-glycine (BDH), pH 8.3, and 1 g of SDS (BDH)/l. Samples (2–50 μg of protein) were precipitated with ice-cold trichloroacetic acid (final concn. 8.3%, w/v), recovered by centrifugation for 5 min at 9000 *g* ($r_{av.}$ = 4.7 cm; MSE Microcentaur), and the pellets were redissolved in 50 μl of 125 mM-Tris/HCl, pH 6.8, containing 20 g of SDS/l, 65 mM-dithiothreitol (Sigma), 140 g of sucrose/l and 10 mg of Bromophenol Blue (BDH)/l. The suspensions were heated for 5 min at 100 °C, centrifuged (9000 *g* for 5 min), and the supernatants applied to the gel. Electrophoresis was carried out for 15 h at 75 V. Proteins were stained with Coomassie Blue R (BDH) (Fairbanks *et al.*, 1971).

Enzymic digestion of proinsulin

Human biosynthetic proinsulin (10–200 μg; gift from Dr. B. H. Frank, Eli Lilly, Indianapolis, IN, U.S.A.) was suspended in 50 mM-Tris/HCl, pH 7.5. Trypsin (12100 BAEE units/mg; Sigma) suspended in the same buffer was added at a ratio of 1:1000 (w/w) relative to proinsulin, and the mixture was incubated in 1.5 ml polypropylene micro-centrifuge tubes (Alpha, Eastleigh, Hants., U.K.) for 5 min at 30 °C (final volume 25 μl). The reaction was terminated by transferring the tubes to a boiling-water bath and heating at 100 °C for 10 min. In some experiments the incubation medium also contained di-isopropyl phosphorofluoridate-treated carboxypeptidase B (90 hippuryl-arginine units/mg; Sigma) at a ratio of 1:20 (w/v) relative to proinsulin.

In experiments to investigate the processing of proinsulin by insulin-secretory-granule carboxypeptidase, the tryptic digest was placed on ice for 15 min and then adjusted to pH 5.5 by the addition of 25 μl of 0.5 M-sodium acetate, pH 5.5. Purified carboxypeptidase in 100 mM-sodium acetate, pH 5.5, was then added at a ratio of 1:100 (w/w) relative to proinsulin, and the mixture incubated at 30 °C for times ranging from 2 to 60 min (final volume 70 μl). Reactions were terminated by the addition of 7.5 μl of acetic acid (BDH).

Then 30 μl of 50 mM-H_3PO_4 (AristaR grade, BDH)/100 mM-$NaClO_4$/10 mM-heptanesulphonic acid (h.p.l.c. grade, Fisons), adjusted to pH 3.0 with NaOH, and containing 25% (v/v) acetonitrile (h.p.l.c. grade S; Rathburn Chemicals), together with acetic acid and acetate buffer if not previously added, was added to each proinsulin digest, and the samples were centrifuged for 5 min at 9000 *g*.

H.p.l.c. analysis of insulin-related peptides

Proinsulin digests (100 μl, containing 10–200 μg) were applied to a 4 mm × 250 mm (3.1 ml) LiChrosorb RP18 h.p.l.c. column (BDH) connected to a f.p.l.c. (fast protein liquid chromatography) system equipped with P3500 booster pump (Pharmacia, Milton Keynes, U.K.). The column was equilibrated in 50 mM-H_3PO_4/100 mM-

$NaClO_4$/10 mM-heptanesulphonic acid, pH 3, containing 33% (v/v) acetonitrile. The column was developed isocratically for 30 min at a flow rate of 1 ml/min, followed by a linear gradient from 33% to 37.5% (v/v) acetonitrile in the same buffer over 60 min. The A_{214} of the eluate was monitored.

For amino acid analysis and gel electrophoresis, the buffer salts were removed by a further chromatographic step. Fractions (1 ml) containing purified (pro)insulin-related peptides were concentrated to approx. 500 μl by freeze-drying (GyroVac; V.A. Howe) to remove acetonitrile, and applied to a 5 mm × 50 mm (1 ml) PepRPC column (Pharmacia) connected to a f.p.l.c. system and equilibrated with 0.1% trifluoroacetic acid (Rathburn Chemicals) containing 26% (v/v) acetonitrile. The column was developed isocratically for 5 min at a flow rate of 1 ml/min, followed by a linear gradient from 26% to 38% (v/v) acetonitrile over 15 min. The A_{214} of the eluate was monitored, and those fractions containing (pro)insulin-related peptides were freeze-dried to dryness.

Alkaline-urea/polyacrylamide-gel electrophoresis

(Pro)insulin-related peptides were separated by alkaline-urea/polyacrylamide-gel electrophoresis, by a modification of the continuous-buffer system of Perrie & Perry (1970). Slab gels (15 cm × 12 cm × 0.15 cm) were polymerized from 150 g of acrylamide/l and 0.9 g of *NN'*-methylenebisacrylamide/l containing 12.5 mM-Tris/80 mM-glycine, pH 8.6, and 6 M-urea. The gels were prerun at 300 V for 3 h, and the upper tank buffer was replaced before samples (2–10 μg) resuspended in 40 μl of 2.5 mM-Tris/HCl, pH 8.6, containing 8 M-urea were loaded. Electrophoresis was carried out for 4 h at 450 V, and the gel was then shaken for 1 h in 25% (v/v) propan-2-ol (BDH), for 2 h in 25% (v/v) propan-2-ol containing 5% (v/v) formaldehyde (BDH) and 1.25 g of Coomassie Blue R/l, and for 4–8 h in 10% (v/v) propan-2-ol containing 10% (v/v) acetic acid.

Amino acid analysis

Insulin-secretory-granule carboxypeptidase (100 μg) was dialysed extensively against deionized water and recovered by freeze-drying. Such fractions (10 μg), and those derived from proinsulin proteolysis, were hydrolysed in 6 M-HCl containing 1 mM-phenol (BDH) (Sanger & Thompson, 1963) for 24, 48 or 72 h at 110 °C *in vacuo*. Amino acids were separated with a Durram auto-analyser (Dionex, Sunnyvale, CA, U.S.A.). Cysteine residues were determined in separate experiments with samples oxidized with performic acid (Hirs, 1967). Tryptophan residues were determined by the spectrophotometric method of Beaven & Holiday (1952).

Chemical deglycosylation

Purified carboxypeptidase (100 μg) was treated with trifluoromethanesulphonic acid (Sigma) by the procedure of Edge *et al.* (1981). The products were subjected to SDS/polyacrylamide-gel electrophoresis, and the electrophoretically separated proteins were transferred to nitrocellulose membranes (Schleicher & Schull, BA85; from Anderman and Co., Kingston-upon-Thames, Surrey, U.K.) by the method of Towbin *et al.* (1979). Concanavalin A binding was detected by the method of Clegg (1982).

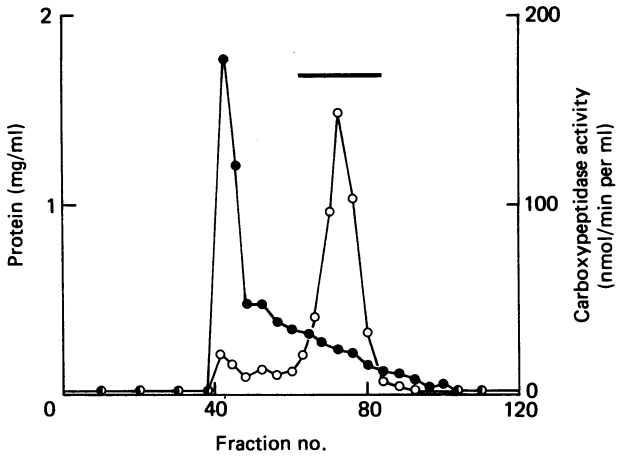

Fig. 1. Gel filtration of partially purified insulin-secretory-granule carboxypeptidase

Partially purified insulin-secretory-granule carboxypeptidase was chromatographed on AcA 44 in buffer A (for details see the text). Fractions (4.6 ml) were assayed for protein (●) and carboxypeptidase activity (○). The fractions combined for further purification are indicated by the horizontal bar.

RESULTS

Purification of insulin-secretory-granule carboxypeptidase

Initial investigations of rat insulinoma fractions prepared by Percoll-density-gradient centrifugation (Hutton *et al.*, 1982) revealed two distinct activities capable of hydrolysing the synthetic substrate Dns-FLR at an acidic pH. One of these, which constituted up to 85% of the total activity, coincided on the density gradient with the lysosomal-enzyme marker arylsulphatase, and was inhibited by 1 mM-phenylmethanesulphonyl fluoride. The remaining activity was localized to the insulin-secretory granules, was unaffected by phenylmethanesulphonyl fluoride, but was inhibited by GEMSA, EDTA, *trans*-1,2-diaminocyclohexanetetra-acetic acid and 1,10-phenanthroline (Docherty & Hutton, 1983; H. W. Davidson & J. C. Hutton, unpublished work).

Further characterization of these activities performed with insulinoma homogenates revealed a marked difference in the stability of the two activities in alkaline media. The lysosomal activity was irreversibly denatured by buffers at pH 8, whereas the secretory-granule activity was stable for over 18 h in the same medium. More than 90% of the secretory-granule activity was recovered in a soluble fraction prepared by hypo-osmotic lysis of purified insulin-secretory granules.

After $(NH_4)_2SO_4$ precipitation of the acidified extract and gel-filtration chromatography, the enzymic activity was eluted as a single peak, with an apparent M_r of 45000 (Fig. 1). The pooled activity peak was then applied to a *p*-aminobenzoylarginine–Sepharose 4B affinity column. The insulin-secretory-granule carboxypeptidase was eluted as a single peak of activity by L-arginine. Under the conditions used, over 90% of the activity loaded bound to the column, of which approx. 90% was recovered in the dialysed eluate (Table 1).

By using the optimized procedure described, the

Table 1. Purification of insulin-secretory-granule carboxypeptidase

Protein was determined by the method of Bradford (1976), and carboxypeptidase activity as described in the text. Activity is expressed in units of μmol of Dns-FLR hydrolysis/min at 37 °C

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	540	12.2	0.02	1.0	100
Extract	431	10.2	0.02	1.0	83
pH 6.5 supernatant	335	9.2	0.03	1.2	75
0–50%-satd.-$(NH_4)_2SO_4$ ppt.	129	7.1	0.06	2.4	58
Pooled AcA44 peak	20	6.1	0.31	13.5	50
Dialysed affinity-column eluate	0.33	5.1	15.5	674	42

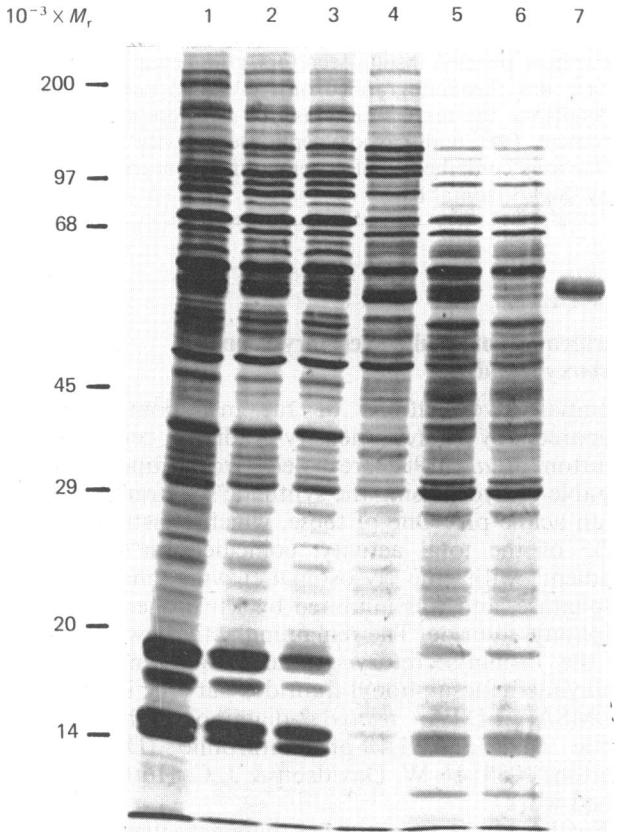

Fig. 2. SDS/polyacrylamide-gel electrophoresis of fractions from the purification of insulin-secretory-granule carboxypeptidase

Fractions from the purification of insulin-secretory-granule carboxypeptidase were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions (for details see the text). Samples are: 1, insulinoma homogenate (50 μg); 2, pH 8 extract (50 μg); 3, acidified extract; 4, $(NH_4)_2SO_4$ precipitate (50 μg); 5, pooled gel-filtration fractions (50 μg); 6, affinity-column non-binding fraction (50 μg); 7, affinity-column eluted fraction (5 μg). M_r values were determined by using reference proteins (SDS-7 and SDS-6H; Sigma).

overall recovery of insulin-secretory-granule carboxypeptidase was 42%, a purification of 674 over the homogenate being achieved (Table 1). SDS/polyacrylamide-gel-electrophoretic analysis of the dialysed eluate under non-reducing or reducing conditions demonstrated a single Coomassie-Blue-staining band with apparent M_r of 55000 (Fig. 2). High-performance gel-filtration chromatography performed on a Superose 12 f.p.l.c. column (Pharmacia) equilibrated with buffer A showed a single A_{280} peak, accounting for over 99% of the protein applied to the column, and having an apparent M_r of 45000 (results not shown).

Molecular properties

The purified enzyme was shown to be a glycoprotein by binding to concanavalin A after transfer to nitrocellulose membrane (Fig. 3). Treatment of the carboxypeptidase with trifluoromethanesulphonic acid eliminated concanavalin A binding, and caused a shift in the apparent M_r on SDS/polyacrylamide-gel electrophoresis from 55000 to 50000, suggesting that carbohydrate comprises approx. 9% of the molecular mass.

Amino acid analysis indicated that the carboxypeptidase contained a relatively high proportion of serine, proline and acidic residues (Table 2). Assuming a methionine content of six residues, the calculated protein M_r was 49866.

N-Terminal analysis by the method of Gray (1972) did not demonstrate a free residue, suggesting that this residue had been modified post-translationally.

Enzymic properties

Synthetic substrates. Kinetic analysis of the purified enzyme gave a K_m towards Dns-FLR of 17.5 μM, with a $V_{max.}$ of 3.4 μmol/min per mg. Inclusion of 1 mM-$CoCl_2$ resulted in a 5-fold increase in $V_{max.}$, without any change in K_m. The enzyme was also stimulated by 1 mM-$NiCl_2$ (1.5-fold), but inhibited by over 90% by $CuCl_2$, $HgCl_2$, $FeSO_4$ and GEMSA (K_i = 7.5 nM). $CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$ all had no significant effect on the rate of hydrolysis.

Analysis of the pH-dependence of Dns-FLR hydrolysis by the purified enzyme revealed an optimum between pH 5 and 6, with little activity below pH 3.5 or above pH 6.5 (Fig. 4). The profile is similar to that previously reported for the impure enzyme with benzoyl-glycyl-arginine as substrate (Docherty & Hutton, 1983).

(Pro)insulin-related peptides. Treatment of proinsulin with trypsin (Fig. 5*a*) resulted in the formation of intermediates identified as diarginyl-insulin (A), [seco-32/33]proinsulin (B) and [seco-65/66]proinsulin (C). Incubation of such tryptic digests of proinsulin with

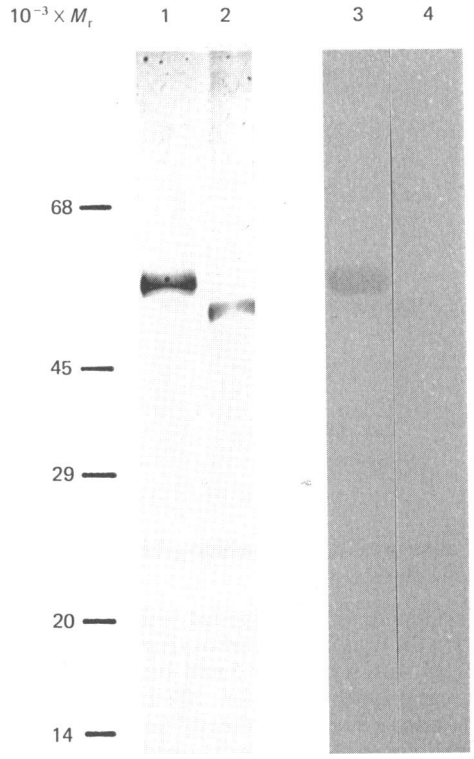

Fig. 3. SDS/polyacrylamide-gel electrophoresis and concanavalin A binding of native and deglycosylated insulin-secretory-granule carboxypeptidase

Insulin-secretory-granule carboxypeptidase was deglycosylated with trifluoromethanesulphonic acid and subjected to SDS/polyacrylamide-gel electrophoresis. One half of the gel was stained with Coomassie Blue (tracks 1 and 2), and the other was transferred electrophoretically on to nitrocellulose membrane and treated with concanavalin A (tracks 3 and 4) (for details see the text). Samples are insulin-secretory-granule carboxypeptidase (5 μg) (tracks 1 and 3), and deglycosylated enzyme (5 μg) (tracks 2 and 4). M_r values were determined by reference to standard proteins (Sigma).

Table 2. Amino acid composition of insulin-secretory-granule carboxypeptidase

Purified insulin-secretory-granule carboxypeptidase (10 μg) was hydrolysed in 6 M-HCl containing 1 mM-phenol, and amino acids were separated by using a Durram analyser (for details see the text). Cysteine was determined in the form of cysteic acid, and tryptophan spectrophotometrically. Values for serine and threonine were extrapolated to zero time.

	Content (mol%)		Content (mol%)
Asx	12.8	Ile	5.2
Thr	5.3	Leu	9.1
Ser	9.1	Tyr	2.8
Glx	11.1	Phe	3.9
Pro	6.1	His	2.7
Gly	7.2	Lys	4.6
Ala	5.7	Arg	5.1
Val	5.7	Cys	1.4
Met	1.3	Trp	1.0

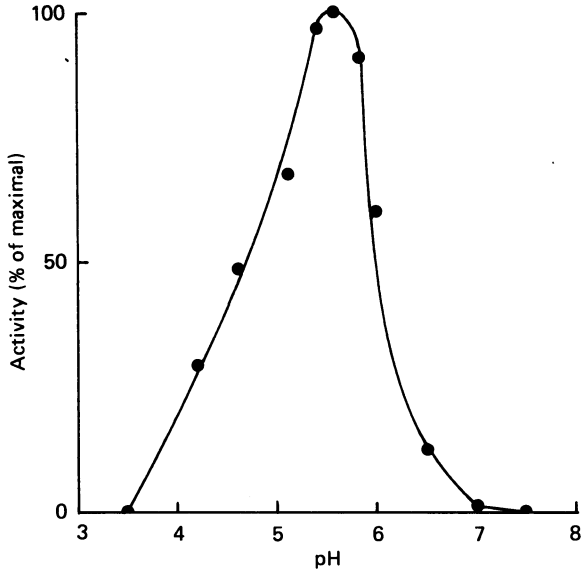

Fig. 4. pH optimum of purified insulin-secretory-granule carboxypeptidase

Carboxypeptidase activity at various pH values was assayed by using Dns-FLR. The buffer used contained 50 mM-sodium acetate (pH 3.5–6.0) or 50 mM-Mes (pH 5.5–8.0) adjusted with HCl or NaOH as appropriate.

purified insulin-secretory-granule carboxypeptidase (Figs. 5*b* and 5*c*) resulted in the time-dependent conversion of these peptides into products identified as insulin (E), des-Arg^{31}, Arg^{32}-proinsulin (F) and des-Lys^{64}, Arg^{65}-proinsulin (G) (Table 3), via intermediates corresponding to monobasic derivatives. Interestingly, the conversion of [seco-65/66]proinsulin into des-Lys^{64}, Arg^{65}-proinsulin proceeded much more rapidly than did the conversion of the two intermediates with *C*-terminal diarginyl residues (Fig. 5*b*). In contrast, the enzyme showed a greater affinity for synthetic substrates with a *C*-terminal arginine than for corresponding substrates with a *C*-terminal lysine (H. W. Davidson & J. C. Hutton, unpublished work).

Electrophoretic analysis of the various digests in a system which separates primarily on the basis of charge confirmed that the fractions prepared by h.p.l.c. each contained a single peptide (Fig. 6*b*). The products of the digests using purified insulin-secretory-granule carboxypeptidase were indistinguishable from those generated in equivalent incubations with carboxypeptidase B (Fig. 6*a*). The change in electrophoretic mobility of the tryptic intermediates after incubation with granule carboxypeptidase was consistent with the removal of basic (positively charged) amino acid residues.

Incubation of either proinsulin or insulin with the purified carboxypeptidase at 30 °C for up to 2 h in buffers of either pH 5.5 or pH 7.5 did not change the h.p.l.c. elution times or gel-electrophoretic mobility of these substrates.

The purified insulin-secretory-granule carboxypeptidase showed a similar inhibitor profile, metal-ion-dependency and pH optimum towards (pro)insulin-related peptides to those exhibited towards synthetic substrates.

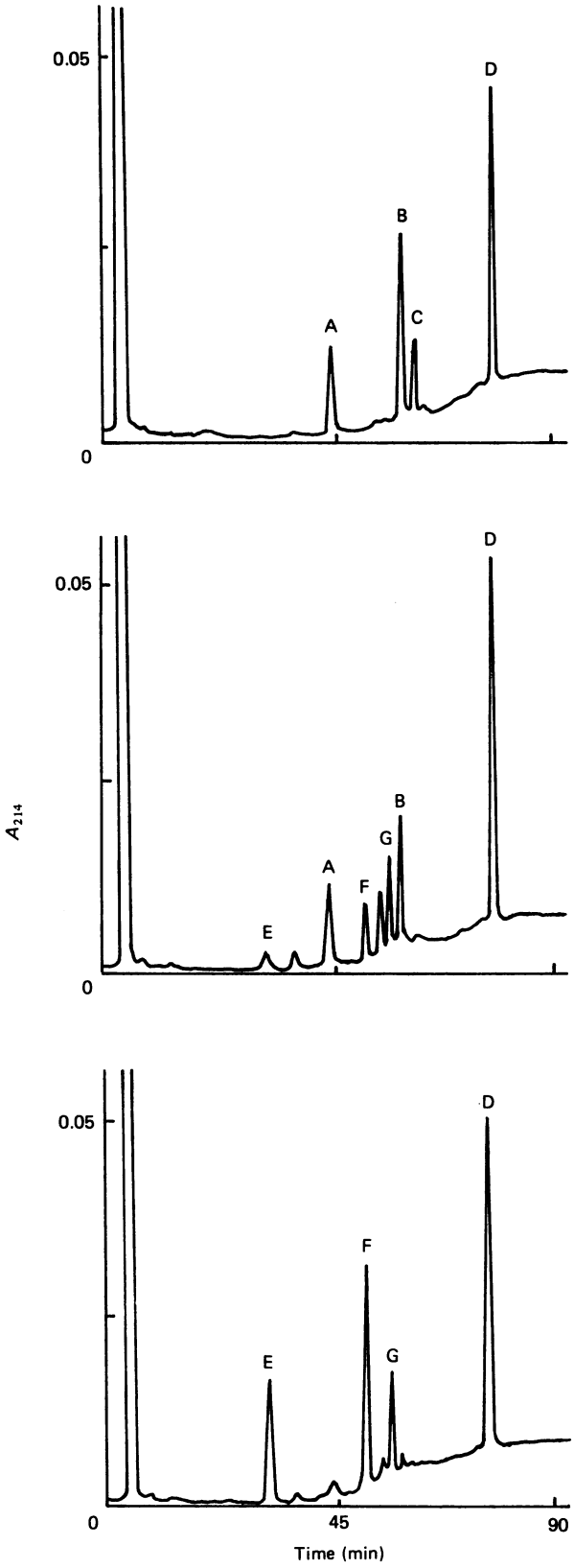

Fig. 5. H.p.l.c. of proinsulin digests

Proinsulin (10 μg) was digested with trypsin for 5 min (*a*), with trypsin for 5 min and insulin-secretory-granule carboxypeptidase for 5 min (*b*), or with trypsin for 5 min and insulin-secretory-granule carboxypeptidase for 30 min (*c*). The products were separated by h.p.l.c. on LiChrosorb RP18 as described in the text.

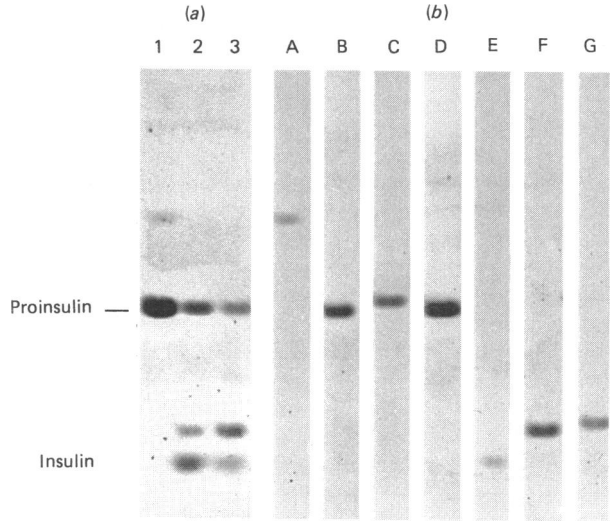

Fig. 6. Alkaline-urea/polyacrylamide-gel electrophoresis of proinsulin digests

(*a*) Proinsulin (10 μg) was digested with trypsin for 5 min (track 1), with trypsin and carboxypeptidase B for 5 min (track 2), or with trypsin for 5 min and insulin-secretory-granule carboxypeptidase for 30 min (track 3). The reactions were terminated by the addition of trichloroacetic acid (final concn. 8.3%, w/v), and the precipitated proteins collected by centrifugation for 5 min at 9000 *g* (MSE Microcentaur). The pellets were resuspended in 40 μl of alkaline-urea sample buffer and separated by alkaline-urea/polyacrylamide-gel electrophoresis as described in the text. (*b*) Purified proinsulin-digestion products (10 μg) prepared by h.p.l.c. (see Fig. 5) were subjected to alkaline-urea/polyacrylamide-gel electrophoresis as described in the text.

DISCUSSION

We have developed a procedure for the isolation of the insulin-secretory-granule carboxypeptidase in high yield. The purified enzyme showed similar properties to activities previously identified in crude secretory-granule fractions (Kemmler *et al.*, 1973; Zuhlke *et al.*, 1977), and was shown by h.p.l.c. and alkaline-urea/polyacrylamide-gel electrophoresis to produce insulin from putative conversion intermediates generated by limited trypsinolysis of proinsulin. The pH optimum of the carboxypeptidase towards either synthetic or polypeptide substrates was in the range 5–6, a value which is consistent with the derived optimum for the overall conversion of proinsulin into insulin (Sorenson *et al.*, 1972; H. W. Davidson & J. C. Hutton, unpublished work) and with the calculated internal pH of the secretory granule (Hutton, 1982).

Analysis of the inhibitor profile, pH optimum and amino acid composition of the purified insulin-secretory-granule carboxypeptidase indicated that it is distinct from carboxypeptidase N (Plummer & Hurwitz, 1978), lysosomal carboxypeptidase B (Ninjoor *et al.*, 1974; Lones *et al.*, 1983), exocrine (pro)carboxypeptidase B (Folk *et al.*, 1960; Cox *et al.*, 1962) and urinary carboxypeptidase (kininase) (Skidgel *et al.*, 1984). However, its properties do resemble those of the enzyme variously termed 'enkephalin convertase', 'carboxypeptidase E' and carboxypeptidase H (EC 3.4.17.10), which

Table 3. Partial amino acid composition of (pro)insulin peptides

(Pro)insulin peptides (10 μg) purified by h.p.l.c. (for details, see the text) were hydrolysed in 6 M-HCl containing 1 mM-phenol for 24 h at 110 °C *in vacuo*, and the amino acid compositions determined with a Durram amino acid analyser. Results are expressed as the number of residues per peptide, assuming an Ile content of 2. Residues which vary in the presence or absence of peptide C (Asx, Gly and Leu), those only present in the insulin molecule (His, Phe and Tyr), and the basic residues involved in processing are shown. The known compositions of proinsulin (P) and insulin (I) are also indicated.

Amino acid / Peptide...	Content (residues/peptide)								
	A	B	C	D	E	F	G	P	I
Arg	3.7	4.8	4.8	4.9	1.0	2.0	3.3	4	1
Asx	3.3	4.2	4.2	4.2	3.0	3.5	3.8	4	3
Gly	4.8	10.8	11.5	10.9	3.3	8.1	10.0	11	4
His	3.0	3.1	3.4	3.1	2.8	2.1	2.9	2	2
Ile	2	2	2	2	2	2	2	2	2
Leu	6.9	13.7	13.2	13.9	6.8	11.5	12.7	12	6
Lys	0.9	1.9	1.8	2.0	0.8	1.5	0.9	2	1
Phe	3.5	3.5	3.1	3.6	3.3	2.0	3.0	3	3
Tyr	4.5	4.6	4.1	4.7	4.5	3.5	4.2	4	4

has been isolated from bovine adrenal medulla, brain and pituitary, and which is probably involved in the post-translational proteolytic processing of prohormones in these tissues (Fricker & Snyder, 1983). This enzyme, like that now purified by us, is also stimulated by $CoCl_2$ and inhibited by GEMSA, and has an acidic pH optimum.

A more detailed comparison between the insulin-secretory-granule carboxypeptidase and 'enkephalin convertase', however, does reveal several differences. For example, there is a variation in the apparent K_m towards the synthetic substrate Dns-FLR [17.5 μM for the insulin-processing enzyme, rather than 70 μM for the adrenal enzyme (Fricker & Snyder, 1983)], and a difference in the extent of maximal $CoCl_2$ stimulation (5-fold and 10-fold respectively). A considerable proportion of 'enkephalin convertase' is integrated into the chromaffin-granule membrane (Supattapone *et al.*, 1984), whereas the pancreatic enzyme appeared to be wholly located in the granule matrix, as demonstrated by the ability to extract more than 90% of the activity in a soluble form without the use of detergents or high concentrations of salt. There are also variations in the inhibitor profiles of the insulin-secretory-granule and the rat pituitary enzyme: the latter is inhibited by both leupeptin and benzylsuccinic acid (Hook & Loh, 1984), neither of which affects the pancreatic enzyme (Docherty & Hutton, 1983; H. W. Davidson & J. C. Hutton, unpublished work).

As noted above, within the brain and adrenal medulla there are two distinct molecular forms of carboxypeptidase H (one being membrane-bound and having an M_r 2500 greater than that of the soluble enzyme) (Supattapone *et al.*, 1984). However, it is noteworthy that, whereas a cDNA clone encoding the bovine enzyme hybridized to three polyadenylated RNAs isolated from bovine pituitary (Fricker *et al.*, 1986), restriction-endonuclease analysis of bovine genomic DNA suggested that only one gene was present. Since the gene apparently contained more than five introns within the coding region for the enzyme, it is possible that multiple enzymic forms might arise from alternative splicing of the heterologous nuclear RNA. Equally they could arise from tissue-specific post-translational modifications, as indicated by the finding that, whereas the bovine adrenal enzyme has a free *N*-terminus (Fricker *et al.*, 1986), the rat pancreatic enzyme *N*-terminus is blocked. Thus it is possible that a family of prohormone carboxypeptidases exists, each member exhibiting subtle variations in its molecular and enzymic properties appropriate to its particular role and environment within different tissues.

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