Preparation and characterization of novel substrates of insulin proteinase (EC 3.4.99.45)

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The specificity of insulin proteinase (EC 3.4.99.45) has been difficult to categorize using only its natural substrates. By exploiting the fact that two substrates competing for the same enzyme inhibit one another, we have found some new substrates of the insulin proteinase from porcine muscle. Two of these substrates, a tryptic fragment of BSA and a fragment of cytochrome *c*, have been shown to be cleaved at a single site. The albumin fragment, as well as another fragment of cytochrome *c*, have susceptibilities ($V_{\max}/K_m$) comparable with that of insulin.

In a second aspect of the study, the porcine-muscle enzyme was shown to be related to other members of its superfamily in that it was immunoprecipitated by a monoclonal antibody raised against the insulin-degrading enzyme from human red blood cells and has the same cleavage sites on insulin as has the rat skeletal-muscle insulin proteinase. We note, however, a possible discrepancy between our results and those of another group regarding the subunit size (110 kDa) of the immunoprecipitated material.

INTRODUCTION

Insulin proteinase (IP, EC 3.4.99.45), also named 'insulinase' or 'insulin-degrading enzyme' has been cloned [1] and shown to be part of a new superfamily of metalloendopeptidases [2,3]. It has been proposed as the proteinase which is responsible for the degradation of insulin after its internalization by cells (see, e.g., [4–6]). The low K_m value of IP for insulin (about 40 nM) supported the hypothesis that the enzyme is specific for insulin. However, it has been reported that the enzyme also degrades other peptide hormones like glucagon [7], the natriuretic-peptide hormones atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide [8], transforming growth factor- α [9], neuropeptide Y and peptide YY [10], whereas other hormones, such as epidermal growth factor, vasoactive intestinal polypeptide and somatostatin [6], are not degraded. This suggests that IP could be important in the processing, and even in the mechanism of action, of peptide hormones [7]. Other studies suggest that IP may be involved in other processes such as the degradation of oxidized haemoglobin [11]. In order to get more information about the specificity of IP *in vitro*, we have tried to find other substrates for IP among the peptides produced by tryptic degradation of BSA and CNBr degradation of cytochrome *c*.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade unless otherwise stated. DEAE-Sephacel and Protein A–Sephacel were purchased from Pharmacia (Uppsala, Sweden). Insulin, ^3H -labelled at position Phe^{B1} (17 Ci/mmol) by a semi-synthetic method described by Halban and Offord [12], was a gift of Dr. J. G. Davies. The cytochrome *c* fragments, prepared as described by Wallace [13], were a gift from Dr. A. Proudfoot. Monoclonal antibody against the insulin-degrading enzyme of human red blood cells was a gift from Dr. R. A. Roth [14].

IP assay

The IP fraction (10 μl) was added to 90 μl of 50 mM Tris, pH 7.5, containing 0.35 % BSA and 50000 d.p.m. of [^3H]Phe^{B1}-insulin. After 10 min at 37 °C, the reaction was stopped by addition of 100 μl of 20 % trichloroacetic acid. The precipitate was removed by centrifugation, and the supernatant was mixed with 5 ml of Picofluor 40 (Packard Instruments, Groningen, The Netherlands) and counted for radioactivity in a Beckman LS 6800 counter. The activity was expressed as percentage solubilization: $100 \times [\text{d.p.m. (supernatant)} - \text{d.p.m. (control without enzyme)}] / (\text{total d.p.m. added})$. A unit of activity is defined as the amount of enzyme which degrades 1 fmol of insulin/min. The ability of a substance to inhibit the degradation of insulin by IP was tested by adding the potential inhibitor to the standard assay and comparing radioactivity solubilization with and without inhibitor: percentage inhibition = $[1 - (\% \text{ solubilization with inhibitor} / \% \text{ solubilization without inhibitor})] \times 100$.

Preparation of IP

Pig skeletal muscle (pork cutlets from a supermarket, which had probably been frozen and thawed at some point before sale) was trimmed of fat and bone, sliced, then homogenized in 2–3 ml of cold phosphate buffer (20 mM sodium phosphate, pH 6.2, containing 0.35 M sucrose)/g of tissue. A blender was used at maximal speed for 4 $\times$ 30 s. The homogenate was stirred magnetically for 10 min, its pH adjusted to 6.2 with 5 M NaOH, and then centrifuged for 40 min at 13000 g. The supernatant was then filtered to remove insoluble fat particles and adjusted to 35 % $(\text{NH}_4)_2\text{SO}_4$ saturation (208 g/l). After 1 h at 4 °C, the precipitate was removed by centrifugation (13000 g, 40 min). Further $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to make it 65 % saturated (210 g/l). After 1 h of magnetic agitation, the pH of the mixture was adjusted to 6.2 with 5 M NaOH just before centrifugation (13000 g, 40 min). The 65 % pellet was redissolved in the phosphate buffer (about 5 % of the starting volume). This 35–65 % fraction was mixed with a suspension of Sepharose 4B

Abbreviations used: IP, insulin proteinase; TFA, trifluoroacetic acid; IC_{50} , concentration giving 50 % inhibition; DTT, dithiothreitol; f.a.b., fast atom bombardment.

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equilibrated with the phosphate buffer (about 1 ml of gel/20 mg of protein). The suspension was adjusted to 45% $(\text{NH}_4)_2\text{SO}_4$ saturation (227 g/l) and agitated for 1 h at 4 °C, after which it was packed in a column containing the same amount of Sepharose 4B equilibrated with phosphate buffer containing 2 M $(\text{NH}_4)_2\text{SO}_4$. The column was then washed with 2 M $(\text{NH}_4)_2\text{SO}_4$ and eluted with a gradient of decreasing salt concentration (2 M to 0 M in the phosphate buffer over 10 column vol.). The active fractions (which were eluted at a conductivity between 140 and 120 mS) were pooled and precipitated with 322 g/l $(\text{NH}_4)_2\text{SO}_4$. The pellet was collected by centrifugation (30 min at 15000 g), dissolved in 50 mM Tris/HCl buffer (pH 7.5) and dialysed twice for 2 h against the same buffer. The dialysis residue was loaded on to a DEAE-Sephacel column (one-fifth of the volume of the Sepharose 4B column) equilibrated with the Tris buffer. The column was eluted with a NaCl gradient (0–500 mM in Tris buffer over 15 column vol.). The active fractions (eluting between 10 and 12 mS) were pooled and concentrated about 20-fold by ultrafiltration on a YM-30 membrane. The concentrated solution, used for peptide digestion, activity tests and for immuno-adsorption, could be kept on ice with the loss of 50% of activity over 2 months.

Immunoprecipitation of enzymic activity

Protein A–Sepharose was washed in Tris buffer, and 50 μl lots of the moist gel were suspended in 100 μl of Tris buffer containing 0, 2, 5 or 10 μg of monoclonal antibody. These samples were gently agitated for 4 h at 4 °C. The gel samples were then washed in Tris buffer by centrifugation, and each was resuspended in 200 μl Tris buffer, containing 7000 units of enzyme. The enzyme solution had been previously agitated with Protein A–Sepharose, 0.5 ml of gel/ml of solution for 4 h at 4 °C, in order to remove any substances that might interfere. The suspensions were gently agitated overnight at 4 °C, and the supernatants separated in a bench centrifuge. The gel pellets were resuspended in 10 vol. of Tris buffer and centrifuged. This washing cycle was repeated three times. The original supernatants, and the washed pellets, were then assayed for enzymic activity as described above. The gel samples were agitated during the assay.

H.p.l.c. analysis

The h.p.l.c. analyses were made on a Nucleosil C-8 column [250 mm $\times$ 4 mm, 30 nm (300 Å) pore size, 5 μm -diameter beads; Macherey–Nagel] at a flow rate of 0.6 ml/min, monitored at 214 nm, and with a solvent system where solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B 0.1% TFA in 90% acetonitrile.

Characterization of peptides

N-terminal sequences were determined by automated Edman degradation on a 477A sequencer from ABI. Fast-atom-bombardment (f.a.b.) m.s. was performed as described by Savoy et al. [15].

Amino acid analysis was performed by acid hydrolysis for 16 h at 120 °C, followed by derivatization with dabsyl (4-dimethylaminoazobenzene-4'-sulphonyl) chloride and h.p.l.c. analysis by the method of Hughes et al. [16].

Tryptic digestion of BSA

BSA (radioimmunoassay grade from Sigma), 1% (w/v) in 1% NH_4HCO_3 was incubated for 18 h at 22 °C after addition of 0.1 mg of tosylphenylalanylchloromethane ('TPCK')-treated

porcine trypsin (Sigma) and 20 μl of CaCl_2 (0.1 M in water)/ml of BSA solution. The digestion was stopped by the addition of 50 μl of 0.1 M phenylmethanesulphonyl fluoride in ethanol.

Purification of BSA tryptic fragments

The digestion mixture obtained above (3 ml) was chromatographed on a Sephadex G-50 (fine) column (45 cm $\times$ 1.5 cm) in 1% NH_4HCO_3 at a flow rate of 0.1 ml/min. The fractions inhibiting insulin degradation were pooled, freeze-dried, redissolved in 1 ml of 0.1% TFA, and further analysed by h.p.l.c. (in 75 μl portions). Fractions corresponding to the peaks observed by u.v. were collected and tested for their ability to inhibit insulin degradation (2 μl /100 μl of the assay mixture described above: the acetonitrile does not interfere).

Reduction of disulphide bridges

Peptides purified by h.p.l.c. were dried in a Speed-Vac apparatus, redissolved in 50 μl of NH_4HCO_3 1% in Milli-Q water and reduced by incubation for 2 h at 37 °C after addition of 50 μl of 0.1 M dithiothreitol (DTT; Suprapur from Calbiochem). The reduction mixtures were then dried in the Speed-Vac apparatus, redissolved in 200 μl of 0.1% TFA and analysed by h.p.l.c..

RESULTS AND DISCUSSION

Two substrates of the same enzyme are expected to compete with each other for the enzyme active site and thus one substrate will act as competitive inhibitor of the reaction of the other one. We have used this fact to develop a strategy for finding new substrates of IP. First, BSA, which does not interfere with the degradation of insulin by IP, was digested under non-denaturing conditions by porcine trypsin. The digestion mixture was then gel-filtered on a Sephadex G-50 (fine) column. While most of the u.v.-absorbing material is eluted as high-molecular-mass species, the IP-inhibiting activity, and thus potential substrates, are eluted with material of low molecular mass. The pooled fractions with inhibiting activity were analysed by h.p.l.c. Figure 1 shows that this pool consist of several peaks. Among the three main peaks

Figure 1 H.p.l.c. purification of the BSA tryptic fragments

The fractions inhibiting insulin degradation collected from the gel-filtration run were further purified by h.p.l.c. (see the Materials and methods section). —, u.v. profile at 214 nm; ----, shape of the eluting gradient (TFA system); 'bars' (i.e. the upper continuous trace), inhibition of insulin degradation by IP.

Figure 2 Reduction of the peptides BSA-23 (a) and BSA-23D (b)

The reduction mixture was analysed by h.p.l.c. (elution gradient: 10 min at 0% solvent B and then a linear gradient to 60% solvent B over 80 min). The elution positions of unreduced BSA-23 and BSA-23D are indicated by arrows.

(nos. 4, 13 and 23 in Figure 1), two (peak 13 and 23) were both able to inhibit insulin degradation and were themselves degraded by IP, whereas peak 4 was neither an inhibitor nor was degraded. Peak 23 (referred to henceforth as 'BSA-23') was chosen for further investigation.

Characterization of BSA-23

BSA-23 was reduced by DTT to give two peaks on h.p.l.c., namely BSA-23a and BSA-23b (Figure 2a). The partial N-terminal sequence of peak BSA-23a was Leu-Phe-Thr-Phe-, and the mass obtained by f.a.b.m.s. was 1851 Da, which permitted the identification of BSA-23a as the fragment Leu⁵⁰³-Lys⁵¹⁸ of the BSA sequence [17]. Amino acid analysis of peak BSA-23a gave results supporting this assignment. In the same way, BSA-23b (N-terminal sequence Thr-Xaa-Met-Glu-, mass by f.a.b.m.s. 3139 Da) was identified as the fragment Thr⁵⁴³-Lys⁵⁷¹ of BSA. In both cases, the C-terminal Lys is compatible with a product of tryptic digestion. According to the disulphide bridge assignment

proposed by Brown for BSA [17], the peptide Leu⁵⁰³-Lys⁵¹⁸ should be connected to Thr⁵⁴³-Lys⁵⁷¹ through a disulphide bridge between Cys⁵¹¹ and Cys⁵⁵⁶ (see Figure 3a), and this is in accordance with our findings.

BSA-23 as substrate of IP

The concentration of BSA-23 giving 50% inhibition (IC_{50}) was determined to be 135 nM by a dose-response curve in the inhibition assay. As this value is expected to be equal to the K_m , this result indicates that the K_m of IP for this new substrate is only about 3 times the K_m reported for insulin. The degradation of insulin (10 μ M) and BSA-23 (2 μ M) with the same amount of IP (about 10000 units/ml in 50 mM Tris, pH 7.5, without BSA) were compared both individually and the two substrates together to estimate the ratio of the V_{max} of both substrates. In the individual assay, the V_{max} obtained for BSA-23 was about half the V_{max} for insulin, while the digestion with both substrates present led to a $V_{max,BSA-23}/V_{max,insulin}$ ratio of 3.4. Both approaches lead to V_{max} values comparable for both substrates. As the K_m values are also comparable, the two substrates must have comparable susceptibilities to degradation by IP.

Cleavage site on BSA-23

The digestion of BSA-23 with IP gave one single peak, BSA-23D, which was eluted 3 min before BSA-23. The reduction of BSA-23D gave two peaks, namely BSA-23Da and BSA-23Db (Figure 2b). BSA-23Da (N-terminal sequence His-Ala-Asp-Ile-, mass by f.a.b.m.s. 1343 Da) was found to be the peptide His⁵⁰⁷-Lys⁵¹⁸ of BSA. The amino acid composition confirmed this structure. BSA-23Db was found to be identical with BSA-23b. The peptide BSA-23D is thus composed of the peptide His⁵⁰⁷-Lys⁵¹⁸ linked by a disulphide bridge (Cys⁵¹¹-Cys⁵⁵⁶) to the peptide Thr⁵⁴³-Lys⁵⁷¹. Thus IP cleaves the peptide BSA-23 at a single site between Phe⁵⁰⁶ and His⁵⁰⁷ (see Figure 3a). The possibility that IP removes the N-terminal amino acids sequentially is unlikely, because no intermediate products were seen during the digestion.

Digestion of cytochrome c fragments with IP

We tested the ability of horse cytochrome *c* and of some of its fragments to inhibit insulin degradation. While cytochrome *c* did not show any detectable inhibitory activity, the peptides cytochrome *c* (Ile⁸¹-Glu¹⁰⁴) (Fig. 3b) and cytochrome *c* [Lys³⁹-Hse

Figure 3 Structure and cleavage sites of (a) BSA-23 and (b) cytochrome *c* (Ile⁸¹-Glu¹⁰⁴)

The sites where IP cleaves these peptides are indicated by arrows.

Table 1 Purification of porcine skeletal-muscle IP

Fraction	Specific activity (units/mg)	Yield (%)	Purification (fold)
Homogenate	8.5	100	1
35–65 %-sald. (NH ₄) ₂ SO ₄	35	185*	4
Sephacrose 4B	2500	380*	290
DEAE-Sephacel	36 000	330*	4200
Immunoprecipitation	3 500 000	ND†	400 000

* We interpret yields of greater than 100% as evidence of enzyme inhibition in the crude fractions.

† ND, not determined.

(homoserine)⁶⁵) did so with IC₅₀ values of 15 and 5 μ M respectively. Digestion of cytochrome *c* (Ile⁸¹–Glu¹⁰⁴) with IP gave two main peaks. Peak 1 (802 Da) corresponds to the peptide Leu⁹⁸–Glu¹⁰⁴, while peak 2 (1993 Da) corresponds to the peptide Ile⁸¹–Tyr⁹⁷. Thus IP cleaves cytochrome *c* (Ile⁸¹–Glu¹⁰⁴) at a single site between Tyr⁹⁷ and Leu⁹⁸ (Figure 3b). Cytochrome *c* (Lys³⁹–Hse⁶⁵), in spite of its *K_m* of about 5 μ M, has a *V_{max}* 35–200 times higher than the *V_{max}* of insulin; it has thus a susceptibility comparable with that of insulin. However, like insulin, BSA-13, neuropeptide Y (*K_m* 0.55 μ M) and peptide YY (*K_m* 0.5 μ M), this peptide was degraded in a more complicated way, giving several degradation products which have not yet been fully characterized.

Porcine skeletal-muscle IP is related to other members of the superfamily

The enzyme prepared as described in the Materials and methods section was immunoabsorbed with an immobilized monoclonal antibody directed against the enzyme isolated from human red blood cells. The amount of enzyme immobilized by immuno-adsorption was dose-dependent with respect to the amount of antibody used, and the enzymic activity was recovered in the gel-bound fraction, showing that the adsorption occurs through epitopes sufficiently distant from the specificity and catalytic sites of the enzyme to permit it to retain its activity. The porcine and human enzymes are therefore immunologically cross-reactive.

The specificity of the enzymic activity with respect to insulin and glucagon was unchanged by immuno-adsorption. Further, the peptide h.p.l.c. profiles obtained were indistinguishable from those obtained previously using soluble rat muscle enzyme both for insulin [15,18–20] and glucagon [7]. The principal sites of cleavage of the pig muscle enzyme were therefore taken to be between the positions A13 and A14, A14 and A15, B9 and B10, B13 and B14, B24 and B25, and B25 and B26 on insulin, and between Arg¹⁷ and Arg¹⁸ on glucagon. We note that immunological and activity relationships have already been shown between a *Drosophila* insulin-degrading enzyme and the rat skeletal-muscle insulin proteinase [21] as well as between the human red-blood-cell insulin-degrading enzyme and the rat skeletal-muscle insulin proteinase [22]. Furthermore, the same degradation products have been found to be generated from insulin by the human fibroblast enzyme [23].

The enzyme prepared here as the main insulin-degrading activity of porcine skeletal muscle differs in its size from the one previously described [24]. Whereas Yokono et al. obtained a

molecular mass of 60 kDa, our immunoabsorbed protein has a molecular mass of 110 kDa as determined by SDS/PAGE (result not shown). This molecular mass is that attributed to the enzyme from human red blood cells [25] and is in agreement with the mass expected from the human cDNA (962 amino acids; 117 890 Da) [1]. The discrepancy with the work of Yokono et al. may be explained by the difference in degree of purification obtained with the two procedures. While their procedure gave a purification factor of 740, the purification factor of the immuno-precipitated enzyme with our procedure is about 400 000 (Table 1). It is thus possible that the band at 60 kDa observed by Yokono et al. was due to a contaminant. Another possible explanation is that the two preparation methods lead to the isolation of two different enzymes. This is, however, unlikely, since the sensitivity of both enzyme preparations to inhibitors are similar and in agreement with the generally accepted sensitivity profile [5]. Our preparation was inhibited by metal chelators, like EDTA (IC₅₀ 2 mM) and 1,10-phenanthroline (IC₅₀ 0.12 mM); by the thiol-blocking agent *N*-ethylmaleimide; by bacitracin (IC₅₀ 0.3 mM) and by transition bivalent cations like Zn²⁺ (IC₅₀ 0.4 mM) and Mn²⁺, Ni²⁺, Co²⁺ and Cu²⁺. Benzamidine, bestatine and phenylmethanesulphonyl fluoride were without effect, while Ca²⁺ and Mg²⁺ acted as activators.

The sequences around the different sites cleaved by IP when acting on a variety of substrates show little or no similarity, except a possible preference for basic or large hydrophobic amino acids in position P'1 [8]. The two cleavage sites described here (Phe–His and Tyr–Leu) are in agreement with this scheme. However, our results show that IP has a preference for small substrates (2–6 kDa). Indeed, tryptic digestion of BSA produces mainly fragments of high molecular mass which are not substrates of IP (no inhibition), while all the inhibiting activity, and thus the substrates, have a low molecular mass. This holds both for the immunoabsorbed enzyme and for the soluble preparation of the enzyme. Previously described substrates (e.g. insulin, glucagon [7] and natriuretic peptides [8]) have molecular masses in this range. The only exception is oxidized haemoglobin [11], which is a partially denatured protein and thus may present a small loop to IP. Similar substrate size recognitions have already been described for other unrelated proteinases, for instance a metalloendopeptidase from *Streptococcus cremoris* H61 [26], and the rat mast-cell proteinase 1 [27].

R.W. thanks Biogen S.A. and Glaxo IMB S.A. for a scholarship. We thank Dr. J. G. Davies for supplying ³H-insulin, Dr. A. Proudfoot for supplying the cytochrome *c* fragments and Dr. R. Roth for the gift of monoclonal antibody against human red-blood-cell insulin-degrading enzyme, and the Swiss National Research Fund for general financial support to this Laboratory.

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Received 16 February 1994/19 April 1994; accepted 4 May 1994