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PARTIAL CHARACTERIZATION OF AN ENDOGENOUS INHIBITOR OF A CALCIUM-DEPENDENT FORM OF INSULIN PROTEASE

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SUMMARY: Insulin protease activity has resisted high-yield purification to homogeneity, due to its low amount in tissues, its instability, and its erratic recovery from several types of chromatography. This report outlines the preliminary characterization of a naturally-occurring insulin protease inhibitor that accounts for some of these problems in rat skeletal muscle. In these experiments, inhibitory activity was assayed by its effect upon hydrolysis of ^{125}I-(A_{14})-insulin by the partially purified insulin protease activity of rat skeletal muscle cytosol. During Sephadex G-200 chromatography of cytosol at pH 7.5, inhibitory activity copurifies with insulin protease activity, and the incomplete resolution of the two activities contributes to the impression that insulin protease exists in distinct 180,000-dalton and 80,000-dalton forms. By contrast, during DEAE-Sephacel chromatography of cytosol at pH 7.5, inhibitory activity and insulin protease activity are resolved by eluting the resin with 50 mM NaCl and 200 mM NaCl, respectively. Post-DEAE-Sephacel inhibitor has an M_r(app) of 67,000 daltons or 80,000-120,000 daltons, as determined by high-performance liquid chromatography or Sephadex G-150 chromatography, respectively. Post-DEAE-Sephacel insulin protease activity exhibits a K_m for insulin of 15 nM and resides in a 200,000-dalton neutral thiol protease which requires 50 micromolar calcium for its maximum insulin-degrading activity. The inhibitor reduces the enzyme's activity reversibly, nonprogressively, and noncompetitively with respect to insulin, but it does not alter the enzyme's sensitivity to calcium ion. These observations suggest that calcium and an endogenous protease inhibitor may influence cellular degradation of insulin via previously unrecognized effects upon cytosolic insulin protease activity.

The enzyme, insulin protease (E.C. 3.4.22.11: insulinase) has been accorded a key role in cell-mediated degradation of insulin (1,2), because it accounts for most of the insulin-degrading enzyme activity of cell extracts (3-5), and because it and intact cells form similar products (3-7) and are inhibited by the same reagents (3-5,8). However, cell-mediated degradation is thought to involve processing of insulin-receptor complexes (9-11) and has a K_m of 10^{-10} M (12,13), while insulin protease has no recognized structural or functional relationship with insulin receptors or involvement in processing events and has a K_m of 10^{-8} M (14,15). Among other things, this paradox may indicate that the properties of insulin pro-

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tease, and its relationship with insulin receptors, are modified during extraction of the enzyme from tissues. By examining this hypothesis, we have discovered an endogenous inhibitor of insulin protease that copurifies with this enzyme and complicates the study of the enzyme's properties and activity in cell extracts.

METHODS:

Preparation of Tissues. Hindlimb skeletal muscle was removed from 200-gram adult male rats (Sprague-Dawley), dissected free from connective tissue and bone, and homogenized at 4°C in a Waring Blendor in 0.35 M sucrose (5 mL/Gm), which contained 50 mM MOPS buffer (pH 7.5), 5 mM EDTA, 2 mM EGTA, and 1 mM DTT. The homogenate was clarified by centrifugation for 20 minutes at 10,000 x g and then was centrifuged for 60 minutes at 100,000 x g ($w^2t = 1.81 \times 10^{10}$ radians^2/sec). Aliquots (25 mL) of the resulting post-microsomal supernatant were chromatographed on a 2.6 x 55 cm bed of Sephadex G-25 Medium (Pharmacia, Piscataway, NJ), which had been equilibrated with 50 mM MOPS buffer (pH 7.5), containing 1 mM EGTA, 1 mM DTT, and the desired concentration of NaCl. A flow rate of 1.0 mL/min was used, and 5-mL fractions were collected. Fractions in the breakthrough peak were pooled and, where necessary, were concentrated by ultrafiltration, using an Amicon PM-10 membrane (Amicon Corp., Lexington, MA).

Column Chromatography. Anion-exchange chromatography was carried out by applying pooled fractions from the G-25 column (35 mL) to a 1.6 x 20 cm bed of DEAE-Sephacel (Pharmacia, Piscataway, NJ), which had been equilibrated with 50 mM MOPS buffer (pH 7.5), containing 1 mM EGTA and 1 mM DTT. Thereafter, the resin was eluted exhaustively with equilibration buffer before increasing the NaCl concentration of this buffer stepwise to 50 mM and then 200 mM. A flow rate of 1 mL/min was maintained and 1 mL fractions were collected.

Molecular sieve chromatography was carried out on 2-mL samples, using a 1.6 x 90 cm bed of Sephadex G-200 or G-150 or of Sepharose 4B, which had been equilibrated with 50 mM MOPS buffer (pH 7.5), containing 1 mM EGTA, 1 mM DTT, and 200 mM NaCl. A flow rate of 0.25 mL/min was maintained and 1-mL fractions were collected.

Hydrophobic chromatography was performed on a 0.9 cm diameter column, containing 3 sample-volumes of phenyl-Sepharose 4B, which had been equilibrated with 50 mM MOPS buffer (pH 7.5), 1 mM EGTA, 1 mM DTT, and 200 mM NaCl. Following application of the sample, the column was eluted with equilibration buffer at a flow rate of 0.1 mL/min and 0.05 x bed-volume (mL) fractions were collected.

High-performance liquid chromatography was performed on a TKS-3000 column (Waters), which had been equilibrated with 10 mM sodium/phosphate buffer (pH 7.5), containing 1 mM EGTA and 1 mM DTT. Following application of a 0.25-mL sample, a flow rate of 1.0 mL/min was maintained, and 1.0-mL fractions were collected.

Polyacrylamide Gel Electrophoresis. Discontinuous nondenaturing electrophoresis of 0.5 x 8.0 cm cyclindrical polyacrylamide gels (7.5% T) was conducted, using the buffer system of Dewald (16), modified by including 1 mM EGTA and 1 mM DTT in all buffers and by omitting detergent from the running buffer. Pre-electrophoresis of resolving gels was carried out at 0.5 mAmp/gel overnight to remove ammonium persulfate from them and to equilibrate them with EGTA and DTT. Then, stacking gels (3.3% T) were photopolymerized in place, using riboflavin as a catalyst. Following electrophoresis, 2 mm slices of gel were placed individually into bovine serum albumin (BSA)-coated tubes, which contained 50 mM MOPS buffer (pH 7.5), 1 mM EGTA, 2 mM $CaCl_2$, and 5 pM ^{125}I-(A_{14})-insulin (10,000 cpm) in a total volume of 1.0 mL. The tubes were incubated for 2 hrs at 37°C and then were assayed for trichloroacetic acid-soluble radioactivity (vide infra).

Assays. Enzyme activity of samples was determined as the increase in trichloroacetic acid-soluble radioactivity (17) during incubation of sample with substrate at 37°C in 50 mM MOPS buffer (pH 7.5), which contained 1 mM EGTA and 2 mM $CaCl_2$. Samples were assayed using ^{125}I-(A_{14})-insulin (5 pM), ^{125}I-glucagon (100 pM), or ^{14}C-methylglobin (5 µG/mL) as substrate. Inhibitory activity of samples was determined by incubating ^{125}I-(A_{14})-insulin (5 pM) with partially purified insulin pro-

tease (10-15 units/mL), in the presence and absence of sample, at 37°C in 50 mM MOPS buffer (pH 7.5), which contained 1 mM EGTA and 2 mM $CaCl_2$ (18). Inhibitory activity (%I) was calculated from the difference in the percentage of ^{125}I-(A_{14})-insulin that was converted to trichloroacetic acid-soluble ^{125}I-products in the presence and absence of sample. All reactions were initiated by adding substrate and were terminated by adding BSA (final concentration 0.25%) and then cold trichloroacetic acid (final concentration 10%). All incubations were carried out in a total volume of 1.0 mL in BSA-coated polypropylene tubes. One unit of enzyme activity was defined as that amount which degraded 1.0 % of substrate per minute.

RESULTS:

Two peaks of insulin-degrading activity were present in the Sephadex G-200 profile of cytosol: a major 180,000-dalton peak and a minor 80,000-120,000-dalton peak (Figure 1). Glucagon-degrading activity cochromatographed with both of these peaks, while globin-degrading activity was eluted ahead of them and between them, at molecular weight values of over 200,000 daltons and approximately 120,000 daltons, respectively. Coeluting with the lower-molecular-weight globin-degrading activity was a broad, asymmetrical peak of inhibitory activity, which presumably distorted the profile of insulin-degrading activity by reducing it in certain column fractions, and possibly by complexing with insulin-degrading enzymes during chromatography. Due to coelution of these various activities, it is unclear how many insulin-degrading enzymes are present in cytosol: two enzymes which differ in molecular weight and globin-

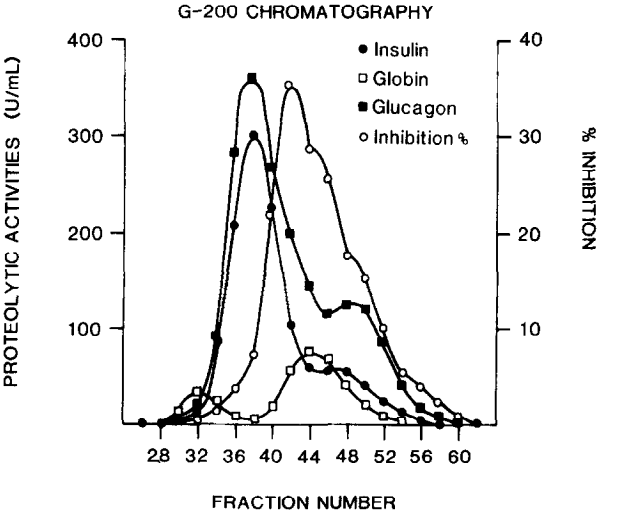

Figure 1: An aliquot of cytosol was desalted on Sephadex G-25 and then was chromatographed on Sephadex G-200. G-200 column fractions were assayed for insulin-, glucagon-, and globin-degrading proteolytic activities and for their ability to inhibit partially purified insulin protease. As indicated, inhibitory activity and all proteolytic activities were eluted in an overlapping fashion between fractions 28 and 60. See text for experimental details and interpretation.

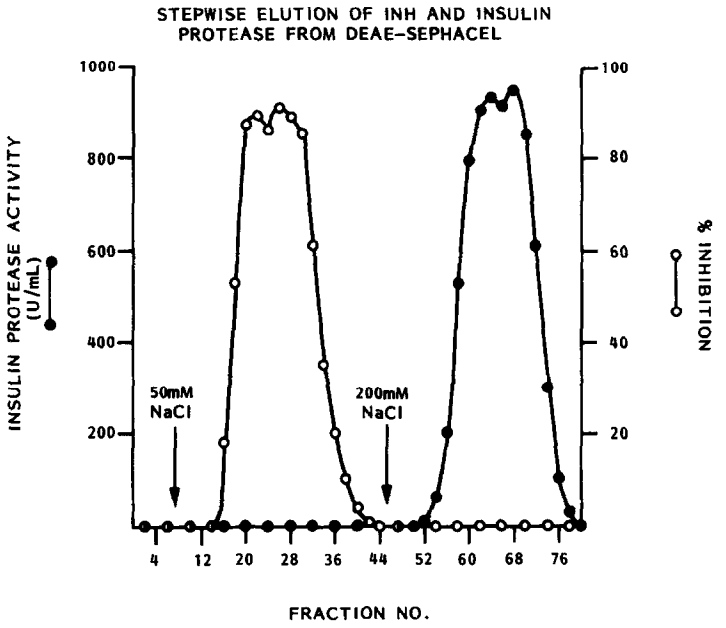

Figure 2: An aliquot of cytosol was desalted on Sephadex G-25 and then was applied to a column of DEAE-Sephacel in a NaCl-free buffer. Unbound proteins were eluted completely prior to fraction 1. At fraction 8, 50 mM NaCl was added to the column buffer and a second peak of protein was eluted between fractions 14 and 40. Thereafter, a third peak of protein was eluted between fractions 52 and 78 by adding 200 mM NaCl to the column buffer at fraction 46. Fractions were assayed for insulin-degrading activity and for their ability to inhibit partially purified insulin protease activity. See text for experimental details and interpretation.

degrading activity, or one enzyme whose apparent molecular weight or catalytic activity is modified by cytosolic inhibitors and/or proteases.

Cytosolic IDA was resolved from inhibitory activity by retaining cytosolic proteins on DEAE-Sephacel in 50 mM MOPS buffer (pH 7.5), which contained 1 mM EGTA and 1 mM DTT (Figure 2). Inhibitory activity was eluted as a single peak by increasing the buffer [NaCl] stepwise to 50 mM. The molecular weight of this inhibitory activity was estimated as 67,000 daltons and 80,000-120,000 daltons by high-performance liquid chromatography and by chromatography on Sephadex G-150, respectively (data not shown). Insulin-degrading activity was eluted from DEAE-Sephacel as a single peak by increasing the buffer [NaCl] from 50 to 200 mM, either stepwise or as a continuous linear gradient. Subsequently, the insulin-degrading activity in this 200 mM NaCl wash was eluted from Sepharose 4B as a single 200,000-dalton peak, which then was eluted from phenyl-Sepharose 4B as a single weakly retarded peak (data not shown). Before and after each chromatographic step, insulin-degrading activity mi-

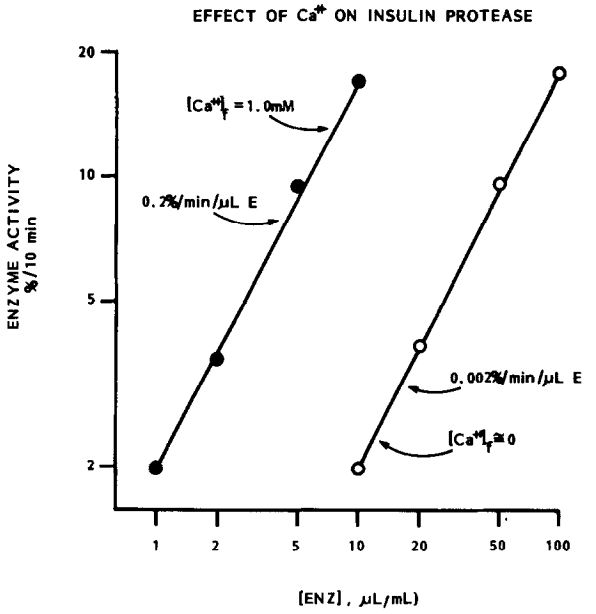

Figure 3: Insulin protease activity was purified by chromatography on DEAE-Sephacel, Sepharose 4B, and phenyl-Sepharose, as described in the text. The insulin-degrading activity of various concentrations of the purified enzyme was then assayed in the presence (closed circles) or absence (open circles) of 1 mM free calcium ion. Enzyme activity was then plotted as a function of enzyme concentration on the log-log scale shown here. See text for experimental details and interpretation.

grated during PAGE as a single species, which was much more calcium-dependent than previous studies would have predicted (14,19). Thus, log-log plots of post-phenyl-Sepharose enzyme activity versus enzyme concentration were linear and parallel in the presence and absence of 1 mM $CaCl_2$, which increased insulin-degrading activity tenfold (Figure 3).

Inhibition of this calcium-dependent post-phenyl-Sepharose insulin-degrading enzyme by post-Sephadex G-150 inhibitor yielded a log %I versus log [I] plot which was linear over its midrange (25-75% I), and which approached 100% I as [I] was increased (Figure 4). Double-reciprocal plots of this enzyme's activity versus insulin concentration, in the presence and absence of inhibitor, demonstrated that the mode of inhibition was noncompetitive and that the enzyme's K_m for ^{125}I-(A_{14})-insulin was roughly 15 nM (Figure 5). The noncompetitive mechanism of inhibition evidently did not involve simple sequestration of calcium ion from the enzyme, since inhibition did not alter the enzyme's marked sensitivity to calcium (full

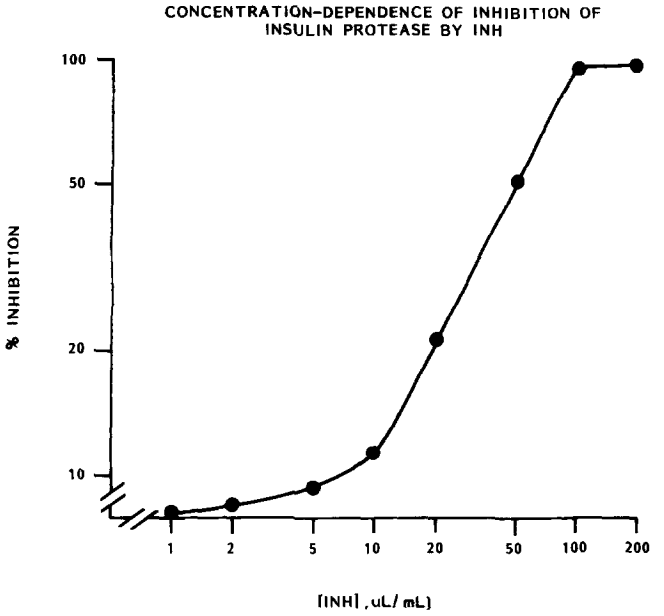

Figure 4: Insulin protease activity was purified by chromatography on DEAE-Sephacel, Sepharose 4B, and phenyl-Sepharose, and the inhibitor was purified by chromatography on DEAE-Sephacel and Sephadex G-150. The insulin-degrading activity of the purified enzyme was assayed in the presence of various concentrations of the purified inhibitor. Percent inhibition of the enzyme then was plotted as a function of the concentration of inhibitor on the log-log scale shown here. See text for explanation of experimental details and interpretation.

activation at 50 uM calcium) and was not reversed by an excess (10 mM) of this ion (Figure 6).

DISCUSSION:

This report outlines the discovery and preliminary characterization of a calcium-dependent form of insulin protease and an endogenous inhibitor of its activity. Copurification of these two species with each other complicates assessment of the enzyme's activity, recovery, fold-purification, physical properties, and contribution to the insulin-degrading activity of tissues. In fact, enzyme-inhibitor interactions may have contributed to the previously encountered resistance of insulin protease to complete purification, due to its low specific activity in tissues, its instability, and its erratic recovery from certain types of chromatography (20). Such interactions may also have been responsible for the apparent resolution of two species of the enzyme in some previous studies (4,19), while others have found only one, whose molecular weight is disputed (14). In theory, interaction with inhibitor

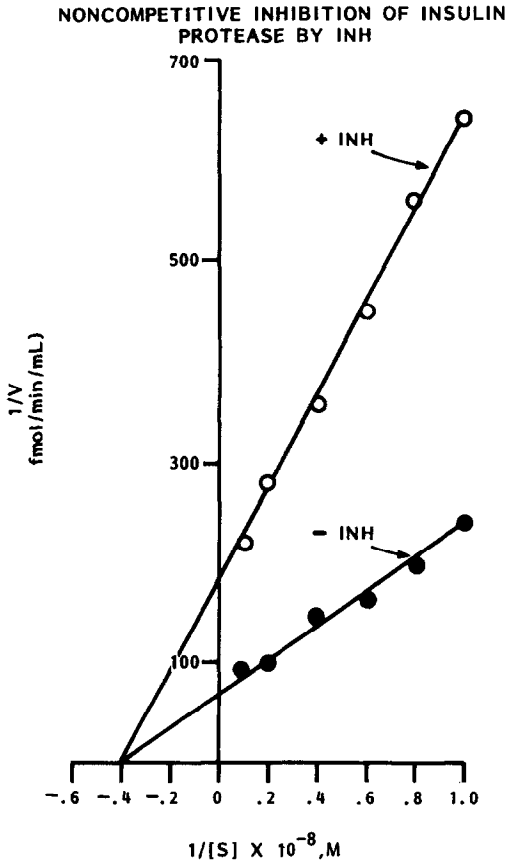

Figure 5: Lineweaver-Burk plots of insulin protease activity as a function of insulin concentration. The insulin-degrading activity (V) of the purified enzyme was assayed in the presence or absence of purified inhibitor at concentrations of insulin (S) ranging from 10^{-7} M to 10^{-9} M. See text for explanation of experimental details and interpretation.

also may have altered the actual or apparent distribution of insulin protease among subcellular compartments (21,22), just as it did here among chromatographic fractions.

Following separation of insulin protease activity and inhibitory activity by anion-exchange chromatography of cytosol, the former has been found in a 200,000-dalton neutral thiol protease, which requires 50 uM calcium for its full insulin- and glucagon-degrading activities. Its inhibitor has been identified tentatively as a high-molecular-weight protein, which reduces the enzyme's activity reversibly and nonprogressively without altering its K_m for insulin or its sensitivity to calcium ion. The true molecular weight of this inhibitor remains in doubt, however, because its elution characteristics are different on different molecular sieves. Ultimately, this difference may be attributable to the inhibitor's non-globular

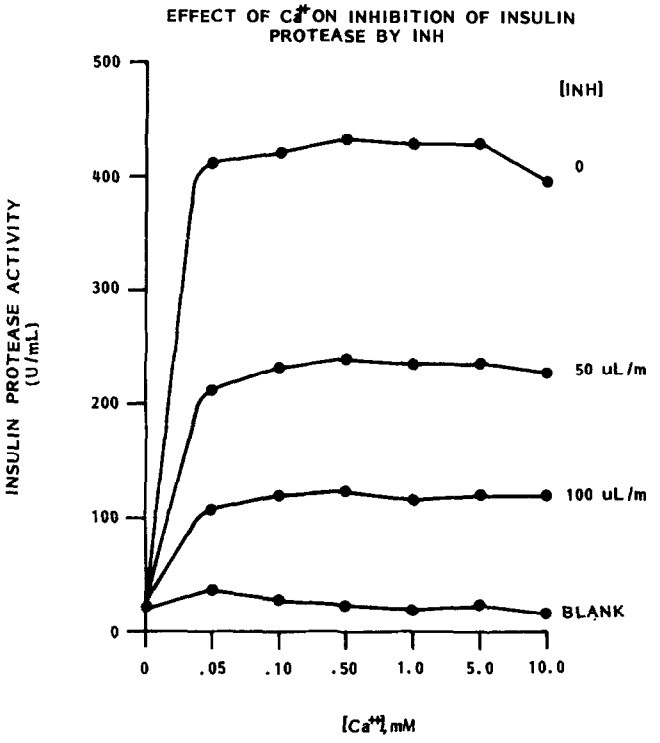

Figure 6: The insulin protease activity of the purified enzyme was assayed in the presence of 0, 50, or 100 uL of purified inhibitor (INH) and at calcium ion concentrations [Ca^{++}] ranging from 0.05 to 10 mM. The purified enzyme was not active in the absence of exogenous calcium ion (0 mM) and the inhibitor alone did not degrade insulin (BLANK). See text for explanation of assay details and interpretation.

or glycoprotein nature or to its aggregation in one of our chromatographic systems but not the other.

The demonstration of a calcium-requiring form of insulin protease suggests that insulin might be degraded by an enzyme similar to those which degrade contractile proteins, intracellular glucoregulatory enzymes, certain hormone receptors, and several polypeptide hormones, including glucagon (23-28). Previous studies may have overlooked this fact, because they measured insulin-degrading activity without regard for its calcium-dependence, or because the calcium-dependent form of insulin protease is not active in crude tissue extracts due to presence of the inhibitor identified here. Whichever the case, conclusions about cell-mediated degradation of insulin drawn from study of cell extracts will need to be re-examined, and the role of specific enzymes re-evaluated, by methods other than the existing catalytic assays of insulin-degrading enzymes. The observations reported here suggest that calcium and an en-

dogenous inhibitor may influence the rate and/or pathway of cell-mediated degradation of insulin via previously unrecognized effects upon cytosolic insulin protease activity.

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