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Isolation and Characterization of an Insulin-Degrading Enzyme from *Drosophila melanogaster*†

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ABSTRACT: An insulin-degrading enzyme (IDE) from the cytoplasm of *Drosophila* Kc cells has been purified and characterized. The purified enzyme is a monomer with an *s* value of 7.2 S, an apparent K_m for porcine insulin of 3 μM, and a specific activity of 3.3 nmol of porcine insulin degraded/(min·mg). N-Terminal sequence analysis of the gel-purified enzyme gave a single, serine-rich sequence. The *Drosophila* IDE shares a number of properties in common with its mammalian counterpart. The enzyme could be specifically affinity-labeled with [^{125}I]insulin, has a molecular weight of 110K, and has a p*I* of 5.3. Although *Drosophila* Kc cells grow at room temperature, the optimal enzyme activity assay conditions parallel those of the mammalian IDE: 37 °C and a pH range of 7–8. The *Drosophila* IDE activity, like the mammalian enzymes, is inhibited by bacitracin and sulfhydryl-specific reagents. Similarly, the *Drosophila* IDE activity is insensitive to glutathione as well as protease inhibitors such as aprotinin and leupeptin. Insulin-like growth factor II, equine insulin, and porcine insulin compete for degradation of [^{125}I]insulin at comparable concentrations (approximately 10^{-6} M), whereas insulin-like growth factor I and the individual A and B chains of insulin are less effective. The high degree of evolutionary conservation between the *Drosophila* and mammalian IDE suggests an important role for this enzyme in the metabolism of insulin and also provides further evidence for the existence of a complete insulin-like system in invertebrate organisms such as *Drosophila*.

Identification and characterization in lower organisms of homologues of mammalian proteins is an approach that can yield information on the role and relative importance of proteins conserved during evolution. One system that appears to be highly conserved between mammals and *Drosophila* is that of insulin and its related proteins. An insulin-like activity in protein extracts from *Drosophila* has been described (Meneses & Ortiz, 1975), and *Drosophila* homologues of the human insulin receptor have recently been identified (Petruzzelli et al., 1985a,b, 1986).

In vertebrates, insulin is an important hormone that has pleiotropic effects on cellular metabolism and growth, including regulation of glucose homeostasis and stimulation of cell proliferation [reviewed in Czech (1985)]. Insulin-induced

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effects are initiated upon binding of insulin to a specific receptor on the surface of cells. The insulin–receptor complex is then internalized by an endocytic pathway leading to degradation of insulin and partial recycling of the receptor (Marshall, 1985a,b; Krupp & Lane, 1982). The actual site of insulin degradation within the cell is not entirely clear. Experiments based upon lysomotropic inhibitors suggest that some degradation of insulin can occur in the lysosomes (Krupp & Lane, 1982; Hammons & Jarett, 1980; Carpenter et al., 1979; Marshall & Olefsky, 1979). In addition, a nonlysosomal insulin-degrading enzyme (IDE)[1] or insulin protease (E.C. 3.4.22.11) has been postulated to initiate insulin breakdown. This cytosolic enzyme, which has been found in a variety of mammalian tissues (Roth et al., 1985; Duckworth et al., 1972; Shii et al., 1986), cleaves insulin at a limited number of sites (Duckworth et al., 1979; Hamel et al., 1986) and generates products that are similar to those found in vivo (Assoian & Tager, 1982; Stentz et al., 1983). The IDE accounts for up to 95% of the degradation of insulin in cell lysates, and reagents that inhibit its activity also inhibit insulin degradation in vivo (Goldstein & Livingston, 1980, 1981). Since the removal of insulin is a critical step in the regulation of the insulin response (Hammons et al., 1982), the possible role of the IDE in the cellular degradation of insulin is of great interest.

We now report the isolation and characterization of an insulin-degrading enzyme (IDE) from the cytoplasm of *Drosophila* Kc cells. The enzyme isolated from *Drosophila* has physical and kinetic properties that are very similar to those of its mammalian counterparts, including molecular weight, isoelectric point, optimal pH for activity, inhibition by sulfhydryl-specific reagents, insensitivity to glutathione, competition by related growth factors, and ability to degrade porcine insulin. The identification of an IDE in *Drosophila* that has been well conserved during evolution suggests an important role for this enzyme in the metabolism of insulin. The fact that in *Drosophila* as well as in mammals there is an insulin-like factor, an insulin receptor, and a cytosolic insulin-degrading activity suggests that these three units are essential components of the insulin system.

Experimental Procedures

Materials. *Drosophila* Kc cells from the MIT tissue culture facility were grown in D22 media. Insulin was purchased from Biomedical Technologies, Inc., Stoughton, MA. [^{125}I]Insulin (88 μCi/μg) was purchased from New England Nuclear, Boston, MA. Bovine serum albumin, β-galactosidase, catalase, alkaline phosphatase, hemoglobin, myosin, phosphorylase *b*, carbonic anhydrase, ovalbumin, *p*-(chloromercuri)benzoic acid, EDTA, EGTA, phenylmethanesulfonyl fluoride (PMSF), aprotinin, bacitracin, benzamidine, leupeptin, pancreatic trypsin inhibitor, *N*-ethylmaleimide, glutathione, Tris, butylagarose, citric acid, diethylbarbituric acid, insulin A and B chains, insulin (porcine and equine), and glucagon were purchased from Sigma Chemical Co., St. Louis, MO. IGF-I was from Toyobo, New York, NY; IGF-II was from Biomedical Technologies, Stoughton, MA. DEAE-Sephadex fast flow, polybuffer exchanger 74, and polybuffer 94 were from Pharmacia, Inc., Piscataway, NJ; hydroxylapatite HTP was from Bio-Rad, Richmond, CA, and ammonium sulfate was from Bethesda Research Laboratories, Bethesda, MD. Inmobilon membranes were from Millipore, Bedford, MA. All other chemicals were reagent grade or the best available. Insulin used for affinity labeling was prepared by using Enzymobeads as previously described (Thompson et al., 1985).

Buffers. Buffer A contained 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.025 trypsin inhibitor unit of aprotinin/mL, 5 μM leupeptin, and 12.5 mM benzamidine. Buffer B contained 30 mM Na_2HPO_4, pH 7.5, 1 mM PMSF, 0.025 trypsin inhibitor unit of aprotinin/mL, 5 μM leupeptin, and 12.5 mM benzamidine. Buffer C contained 25 mM histidine, pH 6.2, 1 mM PMSF, 0.025 trypsin inhibitor unit of aprotinin/mL, 5 μM leupeptin, and 12.5 mM benzamidine. Denaturing buffer (5×) contained 0.5 M Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 10% 2-mercaptoethanol, and 0.5% bromophenol blue. Universal buffer was prepared as described (Davison et al., 1978).

Insulin Degradation Assay. Insulin-degrading activity was determined as described by Shii et al. (1985) with modifications. Briefly, enzyme was incubated in a 100-μL reaction mixture containing 0.1 M Na_2HPO_4 (pH 7.5), 20 μg of BSA, 50 nM insulin, and 1 nM [^{125}I]insulin. After 7 min at 37 °C, 90 μL was removed and mixed with 100 μL of cold 15% trichloroacetic acid. After 15 min on ice, samples were centrifuged at 10000*g* in a Beckman microcentrifuge. Radioactivity in both pellet and supernatant was determined in an LKB RiaGamma counter. One unit of enzyme activity is defined as the amount of enzyme that converts 1 pmol of insulin to acid-soluble material per minute at pH 7.5 and 37 °C.

Purification of the Insulin-Degrading Enzyme. Unless otherwise stated, all operations were carried out at 4 °C. Kc cells (7.2×10^{10} cells) were washed twice in phosphate-buffered saline. The cell pellet was resuspended in 200 mL of buffer A, and the cells were swollen for 15 min. Cells were lysed in a Dounce homogenizer (35 strokes). The lysate was cleared by centrifugation at 20000*g* for 12 min. The supernatant was decanted and the pellet homogenized in 160 mL of buffer A and centrifuged a second time. The combined supernatants were centrifuged at 100000*g* for 90 min. The soluble material [2 g of protein; specific activity, 0.03 nmol/(min·mg)] was loaded onto a DEAE-Sephadex column (2.5 × 19 cm) equilibrated with buffer B. The column was washed with 500 mL of buffer B and the enzyme activity eluted with a 500-mL linear gradient from 0 to 0.3 M NaCl in buffer B. Fractions eluted from the DEAE-Sephadex column containing insulin-binding and -degrading activity were pooled. Solid ammonium sulfate was added to 40% saturation; the solution was stirred for 30 min and centrifuged for 20 min at 15000*g*. The supernatant was adjusted to 80% saturation with ammonium sulfate, stirred, and centrifuged as above. The pellet was resuspended in 10 mL of buffer B with 200 mM sodium chloride and loaded onto a Sephadex G-200 column (50 × 2.6 cm) equilibrated with the same buffer. Fractions containing IDE activity were pooled and loaded directly onto a hydroxylapatite HTP column (9 × 1.5 cm) equilibrated with buffer B. The column was washed with 200 mL of buffer B and the enzyme eluted (at 23 °C) with a linear gradient (200 mL) of 0.03–0.3 M Na_2HPO_4 (pH 7.5) in buffer B. Fractions containing enzyme activity were pooled and adjusted to 40% saturation with ammonium sulfate in buffer B (pH 7.5). After 30 min, the solution was cleared by centrifugation at 20000*g* for 15 min. The supernatant was loaded onto a butylagarose

[1] Abbreviations: IDE, insulin-degrading enzyme; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; *p*-CMB, *p*-(chloromercuri)benzoic acid; PTI, pancreatic trypsin inhibitor; NEM, *N*-ethylmaleimide; TCA, trichloroacetic acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton(s); EGF, epidermal growth factor; HPLC, high-performance liquid chromatography.

column (1 × 9 cm) equilibrated with buffer B containing 40% saturated ammonium sulfate (pH 7.5). The column was washed with 120 mL of the above buffer and the enzyme eluted with a linear gradient (120 mL) of buffer B containing 40–20% saturated ammonium sulfate (pH 7.5). Fractions eluted from the column that contained insulin-degrading activity with highest specific activity were pooled and dialyzed against 30 volumes of buffer C overnight with two changes. Enzyme eluted from the butylagarose column was used for characterization of the properties of the IDE. The dialyzed enzyme was loaded onto a polybuffer exchanger chromatofocusing column equilibrated with polybuffer 74, pH 4. Enzyme was eluted by using the same buffer. Fractions containing IDE were pooled and dialyzed against buffer B or buffer B containing 50% glycerol. Protein was determined according to Bradford (1976). The specific activity of the final enzyme preparation was 3.3 nmol/(min·mg), corresponding to a yield of approximately 1% (0.2 mg of purified protein).

Affinity Labeling of Proteins with [^{125}I]Insulin. Samples from the different columns were incubated with 1 ng of [^{125}I]insulin in the presence or absence of 1 μg of insulin (final volume 54 μL) for 1 h at 4 °C. Disuccinimidyl suberate was added to 0.4 mM and the incubation continued for 15 min. The reaction was stopped by the addition of denaturing buffer. Samples were then heated at 70 °C for 5 min and loaded onto 6.5% polyacrylamide slab gels. Following SDS–PAGE, gels were dried and analyzed by autoradiography as previously described (Thompson et al., 1985).

N-Terminal Sequencing and Amino Acid Composition. Both protein sequencing and amino acid determinations were done by the Harvard University Microsequencing Facility, Cambridge, MA. Samples were prepared by the procedure of Matsudaira (1987) and sequenced on a Applied BioSystems 470 sequencer. A small sample was saved and used to determine amino acid composition using an Applied BioSystems HPLC 130 A system.

Sucrose Density Gradient Ultracentrifugation. Samples (0.2 mL) were layered onto preformed, linear 20–40% sucrose gradients in buffer B. The gradients were centrifuged in a Beckman SW 40.1 rotor at 35000 rpm for 20 h at 4 °C. Fractions (approximately 0.2 mL) were collected from the bottom of the tubes. Catalase, β-galactosidase, alkaline phosphatase, and hemoglobin were included as internal standards.

Determination of Optimal pH for Insulin Degradation. The pH dependence of insulin degradation was determined by substituting the phosphate buffer in the standard assay mixture with universal buffer at different pH values.

Determination of Optimal Temperature for Insulin Degradation. The temperature dependence of insulin degradation was determined by assaying the purified enzyme under standard assay conditions at different temperatures.

Inhibition of [^{125}I]Insulin Degradation by Different Protease Inhibitors and Hormones. Enzyme was incubated with the appropriate concentration of inhibitor for 1 h on ice and assayed as described above. The inhibition by the different hormones was measured by using standard assay conditions except that the appropriate concentration of the hormone was included in the assay mixture.

Results

We initially detected insulin-degrading activity in the cytosol of Kc cell lysates using the trichloroacetic acid (TCA) precipitation assay. This assay, which involves monitoring the increase in soluble counts after precipitation of insulin with cold TCA (Shii et al., 1986), is less sensitive for measuring insulin degradation than other methods such as binding to anti-insulin antibodies (Ryan et al., 1984) or binding to the insulin receptor on fractionated membranes (Roth et al., 1984) or on intact cells (Roth et al., 1985). However, the TCA method has been widely used and shown to give results that are comparable to the receptor binding assay (Roth et al., 1985).

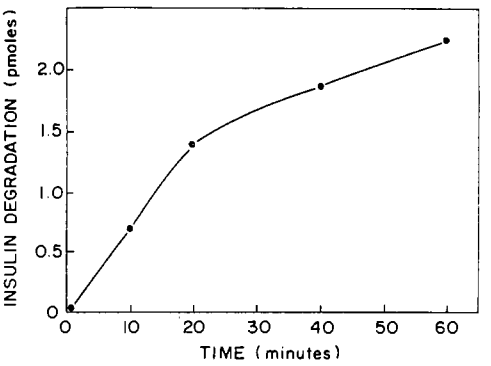

FIGURE 1: Time course of insulin degradation. Purified enzyme (0.4 ng) was incubated with ^{125}I-labeled insulin in a total volume of 500 μL. Samples of 90 μL were taken at different times, and the reaction was stopped with 15% TCA. Samples were then quantitated as described under Experimental Procedures.

Table I: Amino Acid Composition of *Drosophila* IDE

amino acid	μmol	mol %	amino acid	μmol	mol %
Asp[a]	2.101	14.0	Val	0.857	5.7
Glu[b]	1.974	13.1	Met	0.217	1.4
Ser	0.878	5.8	Ile	0.993	6.6
Gly	1.137	7.6	Leu	1.400	9.3
His	0.398	2.6	Phe	0.569	3.8
Arg	0.479	3.2	Lys	0.812	5.4
Thr	0.624	4.2	Cys	nd[c]	nd
Ala	0.839	5.6	Trp	nd	nd
Pro	0.485	3.2		total 15.037	100.0
Tyr	1.275	8.5			

[a] Asp = Asp + Asn. [b] Glu = Glu + Gln. [c] nd = not determined.

Under the conditions of the TCA precipitation assay, the extent of insulin degradation was linear with respect to time for at least 20 min (Figure 1). The degradation of insulin was also linear with respect to protein concentration (data not shown). The specificity of the reaction was illustrated by the fact that addition of excess unlabeled insulin inhibited degradation of the labeled [^{125}I]insulin in the reaction mixture, whereas unrelated proteins such as BSA and ovalbumin had no effect (see Figure 8; data not shown). We chose the TCA precipitation assay to detect the *Drosophila* insulin-degrading activity during purification due to its simplicity and the fact that it is the most rapid of the available assays. The *Drosophila* IDE that we purified using this assay was later shown by HPLC analysis to degrade insulin at a limited number of sites similar to those cleaved by the mammalian IDE (unpublished results).

The insulin-degrading activity was purified from the cytoplasmic fraction of a *Drosophila* Kc cell lysate by successive chromatography on DEAE-Sephadex, Sephadex-G200, hydroxylapatite, butylagarose, and chromatofocusing columns. During chromatography on the DEAE-Sephadex column, two different activities were clearly separated (Figure 2, upper panel). These activities could be differentiated by the fact that the first peak of activity was unaffected by the addition of glutathione to the assay mixture, whereas the second peak of activity was completely inhibited by the addition of 1 μM glutathione (Table I; data not shown). In addition, the first peak contained a 110-kDa protein that could be affinity-labeled

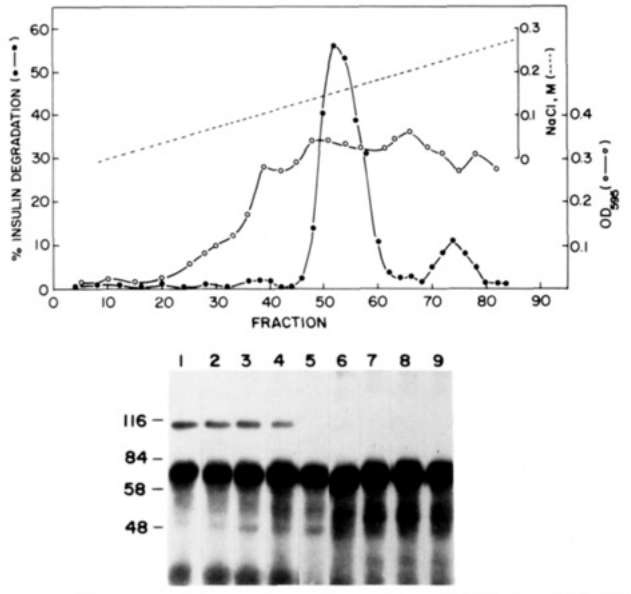

FIGURE 2: Ion-exchange chromatography of IDE on a DEAE-Sephadex column. (Upper panel) IDE activity of eluted fractions. Samples (4 μL/fraction) were assayed in 100-μL reaction mixtures as described under Experimental Procedures. (Lower panel) Affinity labeling of eluted fractions. Samples were incubated with ^{125}I-labeled insulin and cross-linked as described under Experimental Procedures. Lanes 1, 2, 3, 4, 6, 7, 8, and 9 correspond to fractions 48, 50, 52, 60, 30, 72, 74, and 76, respectively. Lane 5 corresponds to fraction 52 incubated as above except that excess unlabeled insulin was included in the incubation mixture. The radioactivity in the 68 000 molecular weight range corresponds to BSA present in the [^{125}I]insulin preparation. Molecular weight markers are indicated ($\times 10^{-3}$).

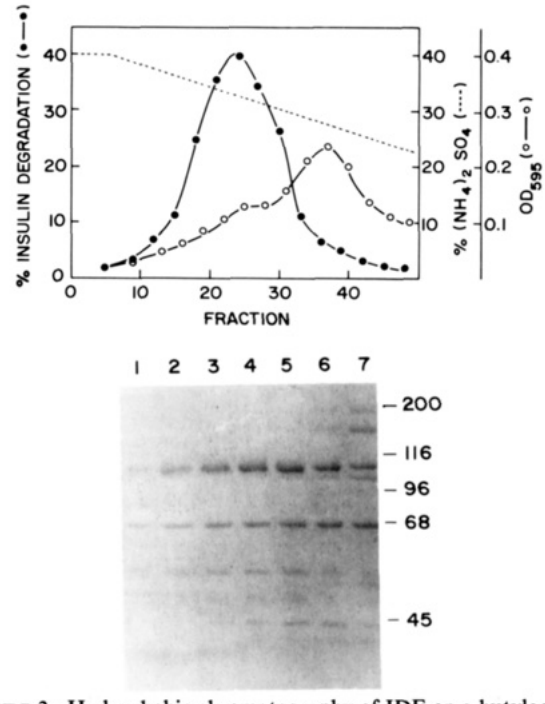

FIGURE 3: Hydrophobic chromatography of IDE on a butylagarose column. (Upper panel) IDE activity of eluted fractions. Samples (4 μL/fraction) were assayed in 100-μL reaction mixtures as described under Experimental Procedures. (Lower panel) SDS–PAGE of eluted fractions. 40 μL each of fractions 10, 15, 18, 21, 24, 27, and 30 (lanes 1–7) was loaded onto a 6.5% gel and stained with Coomassie blue. Molecular weight markers are indicated ($\times 10^{-3}$).

with [^{125}I]insulin, whereas there were no detectable insulin binding proteins in the second peak (Figure 2, lower panel). The first peak of insulin-degrading activity was purified further.

During chromatography on the butylagarose column, the IDE activity eluted as a broad peak (Figure 3, upper panel). When analyzed by SDS–PAGE, fractions with enzyme activity also contained a 110-kDa protein that was affinity-labeled with insulin (Figure 3, lower panel; Figure 5B). Fractions with the highest specific activity from the butylagarose column were pooled and used for the characterization of the physical and enzymatic properties of the *Drosophila* IDE.

Chromatofocusing was used as a final step for purification of the IDE. The enzyme activity eluted as a single peak at pH 5.3 (Figure 4, upper panel). When analyzed by SDS–PAGE on 6.5% gels stained with Coomassie blue, this peak contained one major protein (Figure 4, lower panel, and Figure 5) which had a molecular weight of 110K. Fractions with highest activity for insulin degradation coincided with fractions containing the 110-kDa band. The purified enzyme had a specific activity for porcine insulin degradation of 3.3 nmol/(min·mg), higher than that recently reported for the human erythrocyte IDE (Shii et al., 1986).

Only one protein from the *Drosophila* Kc cell cytoplasmic fraction could be affinity-labeled with [^{125}I]insulin and specifically competed by unlabeled insulin (Figures 2 and 5; data not shown). This 110-kDa protein also copurified with the insulin-degrading enzyme activity at all steps during the purification. The IDEs isolated from several rat tissues and human erythrocytes have a molecular weight of 110K and can also be affinity-labeled with insulin (Shii et al., 1985, 1986). On the basis of these results, we identified the 110-kDa protein as the *Drosophila* insulin-degrading enzyme (IDE).

To determine the amino-terminal sequence and amino acid composition, the *Drosophila* IDE was further resolved by

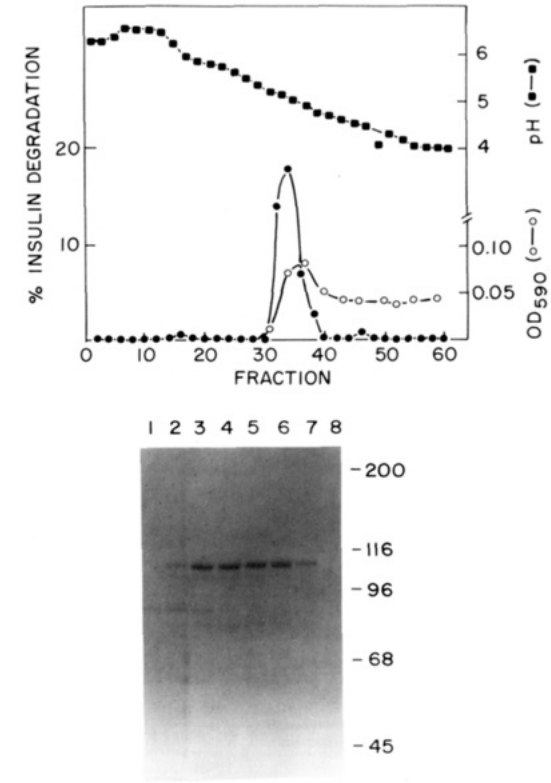

FIGURE 4: Purification of the *Drosophila* IDE by chromatofocusing. (Upper panel) IDE activity of eluted fractions. Samples (10 μL/fraction) were assayed in 100-μL reaction mixtures as described under Experimental Procedures. (Lower panel) SDS–PAGE of eluted fractions. 40 μL each of fractions 40, 38, 36, 35, 34, 33, 32, and 30 (lanes 1–8) was loaded onto a 6.5% gel and stained with Coomassie blue. Molecular weight markers are indicated ($\times 10^{-3}$).

preparative polyacrylamide gel electrophoresis. The purity of the 110-kDa band was confirmed by two-dimensional po-

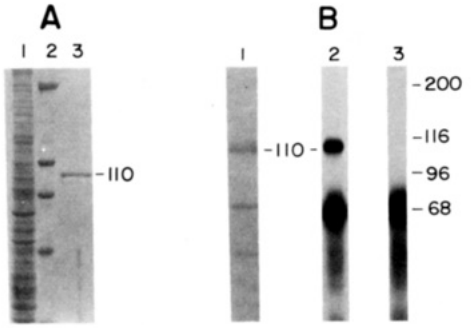

FIGURE 5: SDS–PAGE of the purified IDE and autoradiograph illustrating affinity labeling. For affinity labeling, pooled and dialyzed IDE eluted from the butylagarose column was incubated with ^{125}I-labeled insulin in the presence or absence of unlabeled insulin. Samples were then analyzed by SDS–PAGE as described under Experimental Procedures. (A) Lane 1, total cytoplasmic extract; lane 2, molecular weight marker proteins; lane 3, pooled fractions from the chromatofocusing column. (B) Lane 1, pooled fractions from the butylagarose column; lane 2, IDE affinity-labeled with [^{125}I]insulin; lane 3, IDE affinity-labeled with [^{125}I]insulin in the presence of excess unlabeled insulin. The radioactivity in the 68 000 molecular weight range corresponds to BSA present in the [^{125}I]insulin preparation. Molecular weight markers are indicated ($\times 10^{-3}$).

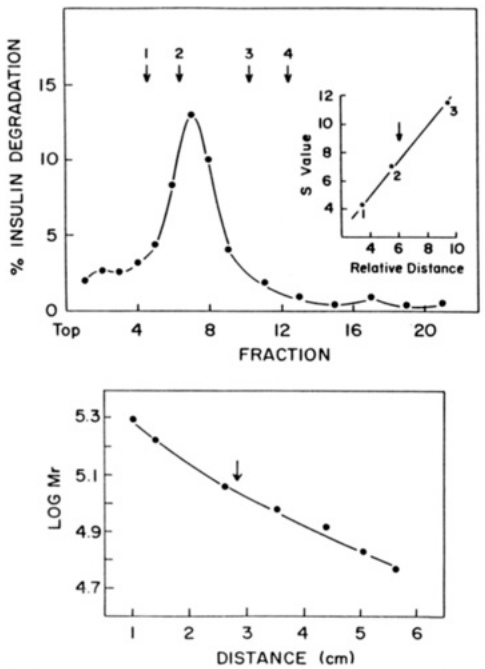

FIGURE 6: Determination of the sedimentation coefficient and molecular weight of the *Drosophila* IDE. (Upper panel) Sedimentation profile of the butylagarose-purified IDE on a 20–40% sucrose gradient. Hemoglobin (1), alkaline phosphatase (2), catalase (3), and β-galactosidase (4) were included as markers. Samples (0.1–0.3 ng/sample) were assayed for IDE activity in 100-μL reaction mixtures as described under Experimental Procedures. (Lower panel) Plot of molecular weight of the purified IDE resolved by SDS–PAGE. Arrow indicates the position of the IDE. Myosin (200K), α_2-macroglobulin (180K), β-galactosidase (116K), phosphorylase *b* (95K), fructose-6-phosphate kinase (84K), bovine serum albumin (68K), and pyruvate kinase (58K) were included as markers.

lyacrylamide gel electrophoresis (Stoppelli, Garcia, and Rosner, unpublished results). For determination of the amino acid composition and N-terminal analysis, the 110-kDa band was blotted onto a membrane filter and then directly analyzed or sequenced. The amino acid composition of the 110-kDa protein is shown in Table I. N-Terminal analysis gave the following sequence: NH_2X-Ile-Ala-Glu-Ser-Ser-Gln-Lys-Ser-Ala-Thr-X-Lys-Pro-Asp (where X refers to an undefined amino acid). No secondary sequences were noted.

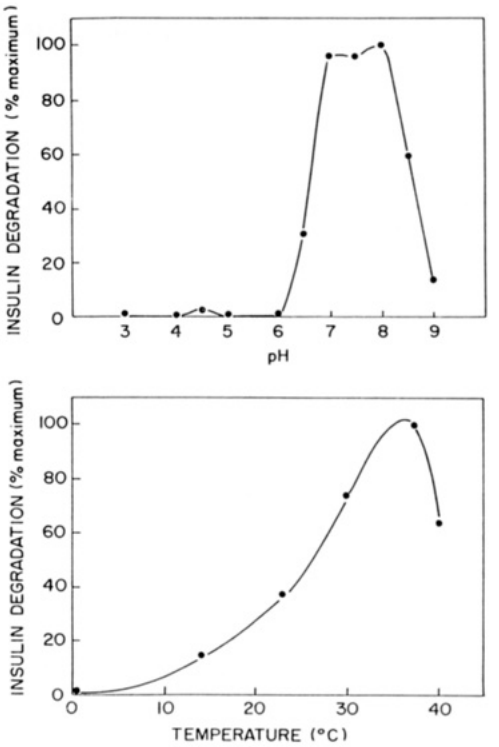

FIGURE 7: Dependence of the *Drosophila* IDE activity on pH and temperature. (Upper panel) pH profile of IDE activity. IDE was assayed in universal buffer at different pH values as described under Experimental Procedures. (Lower panel) Temperature profile of IDE activity. IDE (0.1–0.3 ng/sample) was assayed in 100-μL reaction mixtures as described under Experimental Procedures except that the temperature of incubation was varied.

In order to characterize the *Drosophila* IDE, a number of physical and kinetic parameters were determined. To investigate the subunit structure of the *Drosophila* IDE, the sedimentation coefficient was determined by sucrose gradient centrifugation (Figure 6, upper panel). The sedimentation coefficient of the *Drosophila* IDE was 7.2 S, an *s* value that is in agreement with the 110K determined by SDS–PAGE (Figure 6, lower panel). These results suggest that the *Drosophila* IDE is a monomer, in accord with most of data obtained for mammalian IDEs isolated from different tissues (Roth et al., 1985; Shii et al., 1986; Neal & Kitabchi, 1982).

The optimal pH for the cytosolic *Drosophila* IDE was between 7 and 8 (Figure 7, upper panel). This result correlates well with the optimal pH range for the IDE isolated from human fibroblasts (Stentz et al., 1985) and rat islets (Bhathena et al., 1985). The optimal temperature for assay of the *Drosophila* IDE was close to 37 °C (Figure 7, lower panel), the standard assay temperature for all mammalian IDEs (Duckworth et al., 1972; Roth et al., 1984; Stentz et al., 1985). This temperature is surprisingly high since *Drosophila* Kc cells are grown at 25 °C, which is also the body temperature for the fly. However, there are also several other enzymes isolated from *Drosophila* (Rabin et al., 1986; Foster et al., 1984) that are active at temperatures above 30 °C.

The mammalian IDE has a characteristic sensitivity to sulfhydryl-specific inhibitors (Ryan et al., 1984; Stentz et al., 1985) which is also shared by the *Drosophila* enzyme. As shown in Table II, the *Drosophila* enzyme was inhibited by *N*-ethylmaleimide and *p*-(chloromercuri)benzoic acid, as well as by the antibiotic bacitracin. Like its mammalian counterpart, the *Drosophila* enzyme was not inhibited by protease inhibitors such as pancreatic trypsin inhibitor. While there are conflicting reports on the inhibitory effect of EDTA on

Table II: Effect of Protease Inhibitors on IDE Activity

inhibitor	concn	degradation[a]
p-CMB	1 mM	0
	0.05 mM	53 (5)
EDTA	10 mM	94 (8)
	0.1 mM	99 (4)
aprotinin	0.5 mg/mL	146 (7)
	0.01 mg/mL	117 (6)
bacitracin	1 mg/mL	5 (2)
	0.01 mg/mL	136 (4)
benzamidine	50 mM	49 (1)
	5 mM	100 (3)
leupeptin	0.5 mg/mL	123 (4)
	0.01 mg/mL	115 (7)
PTI	1 mg/mL	133 (12)
	0.01 mg/mL	147 (6)
NEM	1 mg/mL	0
	0.01 mg/mL	48 (16)
glutathione	1 mM	122 (6)
	0.1 mM	113 (8)

[a] Percent of control.

Table III: Similarities between *Drosophila* and Mammalian IDE

	Drosophila	mammalian
subunit M_r	110	110
insulin degradation	+	+
affinity labeling with insulin	+	+
inhibn by sulfhydryl reagents	+	+
inhibn by glutathione	–	–
inhibn by bacitracin	+	+
inhibn by EDTA	–	+/–
optimal pH	7–8	6.5–8.5
isoelectric point	5.3	5.3
assay temp (°C)	37	37
K_m for insulin (μM)	3	0.029–0.13
s value (S)	7.2	nd[a]

[a] Not determined.

the mammalian IDE (Shii et al., 1986; Duckworth & Kitabchi, 1981; Brush, 1971), the *Drosophila* IDE was not inhibited by this chelating agent.

Analysis of the degradation of porcine insulin by the purified *Drosophila* IDE using the TCA precipitation assay yielded an apparent K_m of 3×10^{-6} M and an apparent V_{max} of 2 μmol min^{-1} (mg of protein)$^{-1}$. Although these values are only an approximation due to the relative insensitivity of the assay to single proteolytic cleavages (Duckworth et al., 1979; Hamel et al., 1986), they enable us to compare this enzyme with mammalian enzymes that have been previously characterized. The K_m obtained for the *Drosophila* IDE (Table III) was 1 order or magnitude lower than those obtained for the IDE isolated from rat tissues (Goldstein & Livingston, 1981) and mouse pancreatic acini (Goldfine et al., 1984). It is possible that this discrepancy is due in part to the differences between mammalian insulin and *Drosophila* insulin homologues.

To test the specificity of the IDE, enzyme activity was measured in the presence of increasing amounts of unlabeled insulin-related hormones. Both IGF-2 and equine insulin competed for the degradation of [^{125}I]insulin with an ID_{50} of approximately 10^{-6} M (Figure 8). IGF-1, insulin A chain, and insulin B chain competed with lower affinity (approximately 10-fold). Unrelated proteins like BSA, which is included in the assay mixture, and ovalbumin did not inhibit insulin degradation (data not shown). Mammalian IDE has been shown to degrade insulin-like growth factors and glucagon in addition to insulin (Roth et al., 1984). While the relative pattern of inhibition of the *Drosophila* IDE by these hormones resembles that of the human IDE (Roth et al., 1984), it remains to be determined whether they can be degraded by the *Drosophila* IDE.

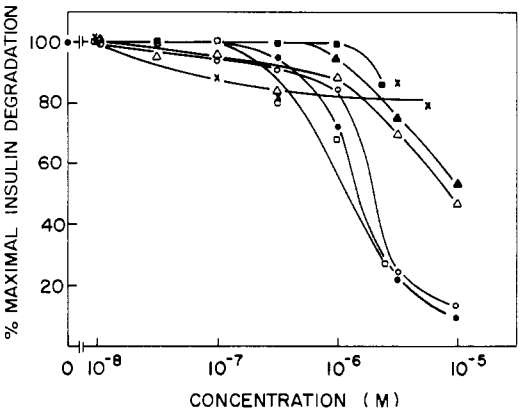

FIGURE 8: Specificity of the *Drosophila* IDE. IDE (0.1–0.3 ng/sample) was incubated with [^{125}I]insulin in the presence of the indicated concentrations of unlabeled ligand: porcine insulin (●); equine insulin (○); IGF-I (■); IGF-2 (□); insulin chain B (▲); insulin chain A (△); glucagon (×). Degradation of insulin was assayed in a 100-μL reaction mixture per sample as described under Experimental Procedures. Each value is the average of two (IGF-1, glucagon) or four determinations (porcine insulin, equine insulin, IGF-2, insulin A chain, and insulin B chain).

Discussion

We report the isolation and characterization of an insulin-degrading enzyme (IDE) from *Drosophila* Kc cells. The purification of the *Drosophila* IDE was facilitated by the use of Kc cells as starting material. These cells contain a relatively high abundance of the enzyme, >10-fold higher than that in the adult fly (Stoppelli and Rosner, unpublished results). The identity of the purified 110-kDa protein as the *Drosophila* IDE was established by several criteria. The final enzyme preparation contained a single 110-kDa polypeptide that correlated with the activity, and the 110-kDa protein was the only one specifically affinity-labeled with insulin. Further, only a single isoelectric point was detected, and only a single amino acid sequence was obtained for the 110-kDa protein, confirming the purity of the isolated IDE.

As summarized in Table III, the properties of the *Drosophila* enzyme are very similar to those of its mammalian counterparts (Roth et al., 1985; Shii et al., 1985; Duckworth et al., 1981). The molecular weight, the isoelectric point, the pH profile, the temperature activity profile, the inhibitor spectrum, and the specific labeling with insulin are all reminiscent of the mammalian IDE. The similarity between the *Drosophila* and mammalian enzymes is further illustrated by the fact that comparable chromatographic steps were used in the purification of the two enzymes, and the final specific activity of the *Drosophila* IDE was higher than that reported for the human erythrocyte IDE (Shii et al., 1986). It is likely that the highly conserved regions are not the most antigenic sites, since monoclonal antibodies that recognize the mammalian rat liver enzyme do not cross-react with the the *Drosophila* IDE and antisera prepared against the *Drosophila* enzyme do not cross-react with the mammalian rat liver IDE (data not shown). The observation that the insulin-degrading enzyme appears to be well conserved during evolution suggests a very important role for the IDE in the metabolism of insulin.

Insulin degradation is a potential control point in the response of cells to insulin, and there is evidence to suggest that the mammalian IDE has a role in the degradation of insulin in the intact cell. Shii and Roth (1986) have demonstrated that microinjection of monoclonal antibodies against the mammalian IDE into cells results in inhibition of insulin degradation by up to 54% relative to control antibodies. More recently, it was shown that intact insulin could be cross-linked

to the IDE in vivo (Hari et al., 1987). However, it is not clear how a soluble cytosolic enzyme might degrade insulin that is internalized by a receptor-mediated pathway. Thus, the intracellular routing of insulin after internalization as well as the relative roles of cytosolic versus lysosomal pathways for insulin degradation remains to be elucidated.

The fact that we have now identified a cytosolic insulin-degrading activity in *Drosophila* suggests that this enzyme is an essential component of the insulin system. Several investigators have reported the presence in *Drosophila* of peptides with bioactivity similar to that of insulin that also cross-react with antibodies against porcine insulin (Meneses & Ortiz, 1975; LeRoith et al., 1981). Recently, a homologue of the human insulin receptor has been isolated from *Drosophila* (Petruzzelli et al., 1985a,b, 1986). The insulin-dependent protein tyrosine kinase from *Drosophila* was found to be developmentally regulated, suggesting a major role for insulin in early *Drosophila* development (Petruzzelli et al., 1985). The *Drosophila* IDE also appears to be developmentally regulated, with highest expression of the degrading enzyme in the later, adult stages of the organism (Stoppelli et al., submitted for publication). Thus, several elements of the insulin system are conserved between mammals and *Drosophila*, and their expression is tightly regulated during fly development.

Drosophila is a good system for studying the function of mammalian growth regulatory proteins since it contains homologues for a number of these proteins. For example, gene sequences related to the retroviral oncogenes *src*, *abl*, and *erb*B have been cloned from the *Drosophila* genome (Simon et al., 1983; Hoffman et al., 1983). Sequence comparison of the *Drosophila* c-*erb*B with human epidermal growth factor (EGF) receptor cDNA indicates there is significant homology in the kinase region and in part of the extracellular domain (Livneh et al., 1985; Wadsworth et al., 1985). Recently, the predicted product of a homeotic gene locus in *Drosophila*, Notch, has been shown to share limited homology with mammalian EGF (Wharton et al., 1985). Finally, transforming growth factor β may be related to the product of the *Drosophila* decapentaplegic gene (Gelbart et al., 1985). Although the function of these *Drosophila* homologues is not known, several have been implicated in the differentiation pathway (Wharton et al., 1985; Gelbart et al., 1985).

Now that the *Drosophila* IDE has been purified, it will be possible to more directly examine the role of this enzyme in the insulin degradation pathway. Using the purified protein, we have generated specific antisera and protein sequence for the isolation of cDNA clones. With these tools, we will be able to study the expression and developmental regulation of the enzyme. Comparison of sequences between the IDE in mammals and these evolutionarily distant organisms will provide important clues for identifying regions involved in the binding and catalytic activity of insulin-degrading enzymes as well as other insulin binding proteins.

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Isolation and Characterization of a Specific Endogenous Na^+,K^+-ATPase Inhibitor from Bovine Adrenal†

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ABSTRACT: In order to identify a specific endogenous Na^+,K^+-ATPase inhibitor which could possibly be related to salt-dependent hypertension, we looked for substances in the methanol extract of bovine whole adrenal which show all of the following properties: (i) inhibitory activity for Na^+,K^+-ATPase; (ii) competitive displacing activity against [^{3}H]ouabain binding to the enzyme; (iii) inhibitory activity for ^{86}Rb uptake into intact human erythrocytes; and (iv) cross-reactivity with sheep anti-digoxin-specific antibody. After stepwise fractionation of the methanol extract of bovine adrenal glands by chromatography on a C_{18} open column, a 0-15% acetonitrile fraction was fractionated by high-performance liquid chromatography on a Zorbax octadecylsilane column. One of the most active fractions in 0-15% acetonitrile was found to exhibit all of the four types of the activities. It was soluble in water and was distinct from various substances which have been known to inhibit Na^+,K^+-ATPase such as unsaturated free fatty acids, lysophosphatidylcholines, vanadate, dihydroxyeicosatrienoic acid, dehydroepiandrosterone sulfate, dopamine, lignan, ascorbic acid, etc. This substance was further purified by using an additional five steps of high-performance liquid chromatography with five different types of columns. Molecular mass was estimated as below 350 by fast atom bombardment mass spectroscopy and ultrafiltration. Heat treatment at 250 °C for 2 h and acid treatment with 6 N HCl at 115 °C for 21 h almost completely destroyed the inhibitory activity of the purified substance for Na^+ pump activity. Additionally, alkaline treatment with 0.2 N NaOH at 23 °C for 2 h destroyed approximately 70% of the inhibitory activity, whereas boiling for 10 min and various enzyme digestion did not destroy the activity. The dose dependency for the four types of the activities for this substance paralleled those of ouabain, spanning 2 orders of magnitude in concentration range. The inhibitory potencies of the purified substance for Na^+,K^+-ATPase, Na^+ pump, and ouabain binding activities were diminished with increasing K^+ concentration, exhibiting a characteristic typical of cardiac glycosides. This substance had no effect on the Ca^{2+}-ATPase activity or the Ca^{2+} loading rate into the vesicle prepared from skeletal muscle sarcoplasmic reticulum. These results strongly suggest that this water-soluble nonpeptidic Na^+,K^+-ATPase inhibitor may be a specific endogenous regulator for the ATPase.

The elevated levels of a humoral factor or factors that inhibit ouabain-sensitive Na^+,K^+-ATPase and Na^+ pump and specific binding of ouabain to the enzyme have been shown in both experimentally hypertensive animals (Buckalew & Nelson, 1974; Huot et al., 1983; Pamnani et al., 1983; Castaneda-Hernandez & Godfraind, 1984; Kojima, 1984; Tamura et al., 1985) and clinically hypertensive subjects (Hamlyn et al., 1982, 1985; Devynck et al., 1983; Grault et al., 1983; Crabos et al., 1984; Graves & Williams, 1984; Vassallo, 1985; Deray et al., 1986). In addition, a similar factor that cross-reacts with anti-digoxin antibodies has also been reported to increase in plasma from various patients with essential hypertension (Deray et al., 1986) chronic renal insufficiency (Kramer et al., 1985; Valdes, 1985) and hypertensive pregnant women (Graves & Williams, 1984; Valdes, 1985), as well as experimental animmals whose extracellular fluid volume was expanded by saline infusion (Gruber et al., 1980) or chronic excessive Na^+ loading (Castaneda-Hernandez & Godfraind, 1984; Kojima, 1984). These observations suggested that there is an endogenous hormone, presumably a specific Na^+,K^+-ATPase inhibitor, that modulates the Na^+ ion transport across

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