Identification of γ-Endorphin-Generating Enzyme as Insulin-Degrading Enzyme†

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ABSTRACT: The EL-4 thymoma cell line contains a peptidase which converts β-endorphin to β-endorphin 1−17 (γ-endorphin), β-endorphin 1−18, and their corresponding C-terminal fragments. This enzyme was purified approximately 700-fold to a single band on an SDS−polyacrylamide gel (106 kDa) in 16% yield. Estimation of the native molecular weight by molecular sieve chromatography gave a value of ∼220 kDa, indicating that this enzyme is a dimer. Peptide sequencing demonstrated this activity can be attributed to insulin degrading enzyme, a previously described member of the inverzincin family (Hooper, 1994). Kinetic studies with a number of peptide substrates indicate that the enzyme preferentially cleaves on the amino side of hydrophobic or basic residues. However, the substrate specificity is more complex since not all basic and hydrophobic residues in a peptide are cleaved. The enzyme exhibits a requirement for a P_2' residue. On the basis of k_{cat}/K_m values, insulin, growth hormone releasing factor, and β-endorphin are nearly equivalent substrates for the enzyme; however, growth hormone releasing factor and β-endorphin exhibit a 40-fold higher k_{cat}, but a 10-fold decreased affinity relative to insulin. A role for insulin-degrading enzyme as both a β-endorphin-processing and -inactivating enzyme is implicated from these studies.

β-Endorphin (1−31) is one of several pro-opiomelanocortin-derived peptides which is released from cells in the pituitary gland as well as in neurons within the central nervous system (Mains et al., 1977; Krieger et al., 1981). Recent studies indicate that β-endorphin, in addition to its opioid activity in the central nervous system (Akil et al., 1984), is involved in the modulation of pituitary hormone release (Howlett & Rees, 1989), the regulation of body temperature (Deeter & Mueller, 1981), and the control of respiratory function (Holaday & Loh, 1981) in the periphery. In addition, β-endorphin appears to be involved in regulating the immune system through both opiate and non-opiate receptor dependent mechanisms. For example, Gilman et al. (1982) demonstrated a proliferative effect of β-endorphin on T-cells, while Heijnen et al. (1986) showed that β-endorphin is involved in the modulation of antibody production. Macrophages secrete β-endorphin (Lolait et al., 1984). In addition, it has been shown that corticotrophin-releasing hormone stimulates the production of immunoreactive β-endorphin by peripheral blood mononuclear cells (Smith et al., 1986), by splenic and mesentric lymph node lymphocytes (Kavelaars et al., 1989), and by $CD14^+$ monocytes (Kavelaars et al., 1990). It has been reported that β-endorphin and its metabolites, α- and γ-endorphin enhance the secretion of IL-2 induced by IL-1 or A23187 (Bessler et al., 1990). Van den Bergh et al. (1994) reported that β-endorphin 1−31 as well as β-endorphin 18−31 enhanced IL-2, IL-4, and interferon-γ production in $CD4^+$ T-cells.

Pharmacological studies have demonstrated the presence of opioid receptors on lymphocytes (Ausiello & Roda, 1984; Mehrishi & Mills, 1983). For example, binding studies have identified the κ-opioid receptor on EL-4 thymoma cells (Fiorica & Spector, 1988) and on P388d macrophages (Carr et al., 1989). However, not all of the effects of β-endorphin, such as stimulation of EL-4 cell proliferation (Schweigerer et al., 1985), are blocked by the opioid antagonist naloxone. It has therefore been suggested that β-endorphin and its metabolites also act through a non-opioid receptor mechanism (Schweigerer et al., 1985; Hazum et al., 1979; Mandler et al., 1986; Shahabi et al., 1990), and a receptor, referred to as the "ϵ" receptor, has been postulated to play a role in these processes (Schweigerer et al., 1985; Hazum et al., 1979).

The enzyme(s) involved in processing β-endorphin have not been previously purified, although such activities have been reported (Lebouille et al., 1986; Miller et al., 1995). The EL-4 thymoma cell line and anti-CD3 antibody activated murine $CD4^+$ spleen T-cells metabolize β-endorphin to β-endorphin 1−17 (γ-endorphin) and β-endorphin 1−18 (Miller et al., 1995). In this report we demonstrate that this γ-endorphin-generating enzyme is identical to insulin-degrading enzyme (IDE, EC 3.4.24.56).

MATERIALS AND METHODS

Chemicals. Human β-endorphin (1−31), γ-endorphin (β-endorphin 1−17), dynorphins A (1−5), (1−7), (1−8), (1−9), (1−10), (1−13), (1−17), α-neo-endorphin, pancreastatin, and human adrenocorticotropic hormone (ACTH 1−24) were purchased from Bachem (California). Substance P (1−6) and (1−7) were from Peninsula Laboratories. Nitroblue tetrazolium was obtained from Bio-Rad. 5-Bromo-4-chloro-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecysulfate polyacrylamide gel electrophoresis; GRF, growth hormone releasing factor; HPLC, high-performance liquid chromatography; NEM, *N*-ethylmaleimide; PVDF, polyvinylidene difluoride; Tween 20, polyoxyethylene sorbitan monolaurate; PVP-40, polyvinylpyrrolidone.

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3-indolyl phosphate was obtained from Boehringer Mannheim. Galanin was a gift from Dr. Georgetta Howard, University of Kentucky. All other reagents and peptides were purchased from Sigma Chemical Co. unless otherwise specified.

Measurement of β-Endorphin Endopeptidase Activity. β-Endorphin endopeptidase activity was followed by measuring either the disappearance of β-endorphin or the appearance of γ-endorphin by automated reverse-phase HPLC. Each reaction contained 30 μM β-endorphin, 50 mM NaCl, 80 mM Tris-HCl, pH 7.5, and enzyme in a final volume of 100 μL. Reactions were started by the addition of enzyme and incubated at 37 °C. At the desired time the reaction was terminated by the addition of 10 μL of 5% trifluoroacetic acid and boiled for 2 min. An aliquot of the reaction (95 μL) was injected on to a Vydac C-4 reverse phase column and eluted with a 60 min linear gradient of 5−75% acetonitrile containing 0.10% trifluoroacetic acid, at a flow rate of 1 mL/min. Peptides were detected at 214 nm and quantitated by determining their peak area. Standard curves were constructed for β-endorphin and γ-endorphin, the latter identified by comparison of its elution time with an authentic standard.

Endopeptidase Purification. EL-4 cells were subcultured at an initial concentration of 0.25×10^6 cells per mL for 2−3 days in 8 L of RPMI 1640 plus 10% fetal bovine serum (FBS). Cells were harvested by centrifugation, washed with Hank's salt solution, and resuspended in Hank's salt solution at a concentration of 5×10^6 cell per mL, ∼4 L. Cells were incubated for 2−3 days at 37 °C, 10% CO_2, to permit secretion of the enzyme (Miller et al., 1995). The supernatant was harvested by centrifugation and concentrated/dialyzed using an Amicon concentrator with a YM 30 membrane. The media was first concentrated to ∼50 mL, then diluted to 500 mL with 40 mM Tris-HCl, pH 7.5, and concentrated to approximately 100 mL. This procedure was repeated a second time, after which the concentrated solution was stored frozen until used.

The concentrated/desalted supernatant was thawed and centrifuged at 40 000*g* for 20 min to remove denatured proteins and other cell debris. The supernatant was loaded on to a 15 mL Accell Plus QMA anion exchange column previously equilibrated with 40 mM Tris-HCl buffer, pH 7.5. The column was washed with 4 column volumes of the equilibration buffer, and the enzyme was then eluted with a linear gradient from 0 to 0.4 M NaCl. β-Endorphin endopeptidase activity was eluted at 0.25 M salt. Active fractions were pooled, adjusted to 20% ammonium sulfate in 40 mM Tris-HCl buffer, pH 7.5, and loaded on to a 35 mL Toyopearl Butyl-650S column (ToSoHAAS). After the column was washed with 2 column volumes of starting buffer, the enzyme was eluted by a decreasing linear gradient to 0% ammonium sulfate. The endopeptidase was eluted at approximately 16% ammonium sulfate. Active fractions were pooled, concentrated to 2 mL, and loaded on to a 12 × 60 cm gel-filtration column (Superdex-200) equilibrated and eluted with 40 mM Tris-HCl buffer, pH 7.5.

Peptide Sequencing. 100 μg of purified secreted γ-endorphin-generating enzyme was electrophoresed on a 10% SDS−PAGE and transferred to PVDF paper, using Tris-Glycine buffer, containing 10% methanol (Towbin et al., 1979). The band was identified by incubating the PVDF paper in 0.1% Ponceau S solution prepared in 10% acetic acid. The band was excised and treated with endoproteinase Lys C (Fernandez et al., 1994). Peptides were separated on a C-4 column using HPLC and sequenced at the Molecular Structure Analysis Facility of the University of Kentucky.

Antibody Generation. 1 mg of partially purified β-endorphin-cleaving enzyme was electrophoresed onto a 10% SDS−PAGE, and the gel was washed 3 times with deionized water. The band of interest was located by the incubation of the gel in 0.05% Coomassie Brilliant Blue R-250 prepared in water and excised. A rabbit was injected with ∼300 μg of enzyme protein subcutaneously 14, 21, and 56 days after the initial inoculation with an emulsion of SDS−PAGE and Freund's complete adjuvant. On the 56th day, a piece of PVDF paper containing 30 μg of purified protein was implanted in the popliteal and inguinal glands in addition to the SDS−PAGE booster shot.

Western Blotting. Proteins were separated on a 10% SDS−PAGE and transferred to PVDF paper as described for peptide sequencing (above). Membranes were blocked with 138 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5% Tween 20, 1% PVP-40, and 5% nonfat dry milk as described by Heycock (1993). Membranes were incubated in this buffer for 1 h, and the antiserum at 1:200 dilution was then added and left overnight. The membranes were washed 3 times, 10 min each time with the above buffer minus milk and PVP-40. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used at a dilution of 1:2000 in the above buffer for 1 h and after washing the membrane 3 times as before, the membranes were incubated in a buffer of 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $MgCl_2$, 0.3 mg of nitroblue tetrazolium/mL and 0.15 mgof 5-bromo-4-chloro-3-indolyl phosphate mL. The enzyme was visualized as a bluish-purple band.

Determination of Kinetic Constants and Bond Cleavages. The hydrolysis of β-endorphin was measured by following the appearance of γ-endorphin as described above. Reaction mixtures contained 80 mM Tris-HCl (pH 7.5), 50 mM NaCl, enzyme, and varying concentrations of β-endorphin (10−50 μM). Reactions were started by the addition of enzyme and incubated at 37 °C for 20 min. Preliminary experiments were used to determine that under these conditions the rate of hydrolysis is linear between 10−100 μM of β-endorphin for at least 30 min. K_m values for other peptides were determined by using them as alternate substrate inhibitors at a fixed β-endorphin concentration (13 μM) equivalent to its K_m. Under these conditions, the apparent K_i is equivalent to $K_m/2$.

The rate of hydrolysis of other peptides was determined in a similar manner by following the disappearance of the parent peptide by reverse-phase HPLC. Each reaction contained 100 μM of the peptide substrate, 50 mM NaCl, 80 mM Tris-HCl, pH 7.5, and purified enzyme in a final volume of 100 μL. Reactions were incubated at 37 °C for various times, and the reaction was terminated as described above. Peptide separation by HPLC was achieved using a linear gradient of 5−75% acetonitrile containing 0.10% trifluoroacetic acid at a flow rate of 1 mL/min. The linear portion of each reaction was used to determine the rate of hydrolysis. For k_{cat} values, a molecular mass of 106 was used. To determine cleavage sites products were collected and their N-terminal sequence determined by automated Edman degradation using an on-line Applied Biosystems model 120A system.

Table 1: Purification of β-Endorphin Endopeptidase Activity[a]

purification step	total protein (mg)	total activity (nmol/min)	specific activity (nmol/min/mg)	recovery (%)	*n*-fold Purification (*n*)
cell supernatant	123	111	0.9	(100)	1
ion exchange chromatography	7.5	45	6	40	7
hydrophobic chromatography	0.14	35	248	32	276
gel filtration chromatography	0.03	18	600	16	667

[a] β-Endorphin-cleaving activity was determined by following γ-endorphin formation as described in Materials and Methods. Protein was determined with the Coomassie Plus Protein Assay Reagent of Pierce Chemical Co. using bovine serum albumin as a standard.

RESULTS

Purification of β-Endorphin Endopeptidase. The secreted β-endorphin degrading endopeptidase was purified from EL-4 cells in a three-step procedure, starting with chromatography on an anion-exchange column, followed by hydrophobic chromatography and molecular sieve chromatography, Table 1 and Figure 1. Starting with 2×10^{10} cells yielding 123 mg of protein, 30 μg of purified enzyme was obtained in 16% yield, with an overall purification of ~700-fold. It can be estimated from the data in Table 1 that ~0.14% of the secreted proteins represent this endopeptidase. The purified enzyme exhibited a single protein band when analyzed by SDS−PAGE, Figure 2, with a molecular mass of 106 kDa. A value of 220 kDa was obtained for the molecular mass of the native enzyme as estimated by gel filtration, indicating the enzyme is a dimer, Figure 3.

Analysis of β-endorphin metabolism by the peptidase revealed β-endorphin 1−17 (γ-endorphin) and β-endorphin 1−18 as the major metabolites. These are the same products produced by a β-endorphin-metabolizing enzyme referred to as the γ-endorphin-generating endopeptidase (Lebouille et al., 1989). There was some further metabolism of these products in unfractionated media, however, but after the first anion exchange chromatography step γ-endorphin formation was linear and stable and was used as a measure of enzyme activity. Therefore, the activity in Table 1 reported for the secreted enzyme is most likely an underestimate of the total activity.

Identification of the β-Endorphin Endopeptidase As Insulin-Degrading Enzyme. 100 μg of purified secreted β-endorphin cleaving enzyme (γ-endorphin generating endopeptidase) was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to PVDF paper. The section of the paper containing β-endorphin cleaving enzyme was digested with endoproteinase Lys C. The N-terminal sequences of three resultant peptides were obtained. These peptides showed 100% identity to the rat insulin-degrading enzyme (IDE) sequence, Figure 4.

Characterization of the Secreted β-Endorphin Endopeptidase/Insulin-Degrading Enzyme. The secreted EL-4 endopeptidase was inhibited by the metal chelators 1,10-phenanthroline (100% at 1.0 mM), EDTA (77% at 1.0 mM), and NEM (100% at 50 μM). These findings are consistent with the previously reported inhibition data on both γ-endorphin-generating enzyme and insulin-degrading endopeptidase (Lebouille et al., 1989; Shii et al., 1986).

With β-endorphin as substrate, the EL-4 endopeptidase showed highest activity between pH 6.9 and 7.5 for cleavage at both the Leu-Phe bond (γ-endorphin formation) and the Phe-Lys bond (β-endorphin 1−18 formation), Figure 5. This is consistent with the pH-rate profile that has been reported for insulin degrading endopeptidase from other sources (Shii et al., 1994; Stentz et al., 1985). However, with dynorphin B 1−13 as substrate, where cleavage occurs between an Arg-Arg bond (see below), the endopeptidase activity remains constant and maximal out to pH 9.2, Figure 5.

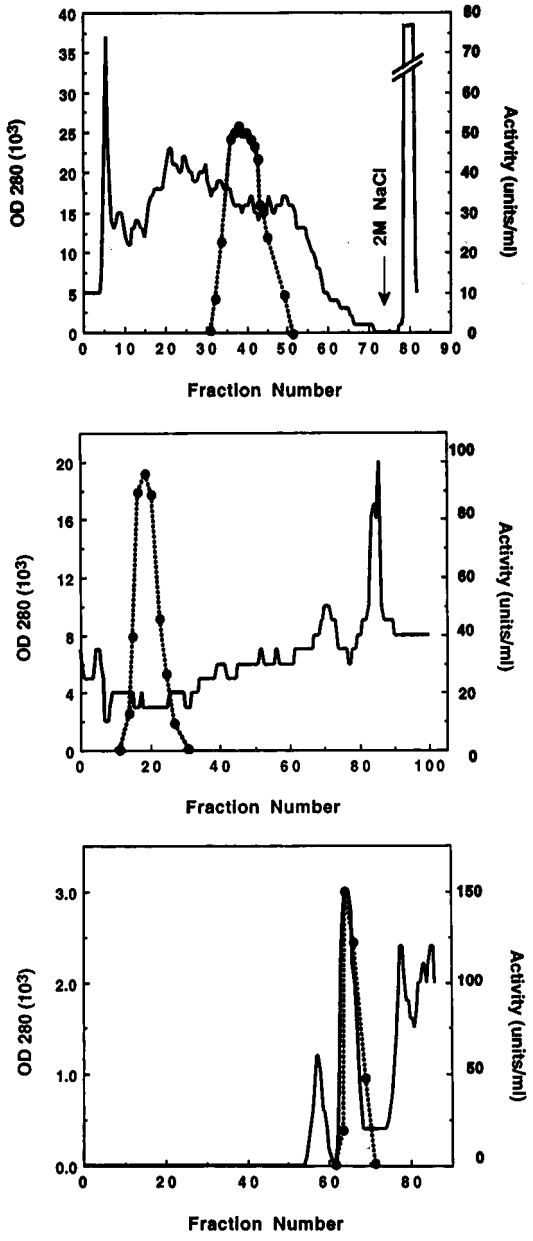

FIGURE 1: Elution profiles of β-endorphin endopeptidase. β-Endorphin endopeptidase was purified as described in Materials and Methods using Accell Plus QMA column (Top), Toyopearl Butyl-650S column (middle), and Superdex-200 gel-filtration column (bottom). One unit of activity is equal to 160 pmol of γ-endorphin generated/hour (- - -). The full-scale absorbance was set at 0.1 for the first column, 0.05 for the second column, and 0.02 for the third column.

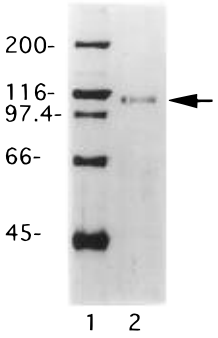

FIGURE 2: SDS−PAGE analysis of purified β-endorphin endopeptidase. 1.5 μg of the endopeptidase was electrophoresed (lane 2) along with molecular weight standards (lane 1) on a 10% acrylamide-SDS gel according to the procedure of Hames (1981). Protein was visualized by silver staining according to the method of Gottlieb and Chavko (1987).

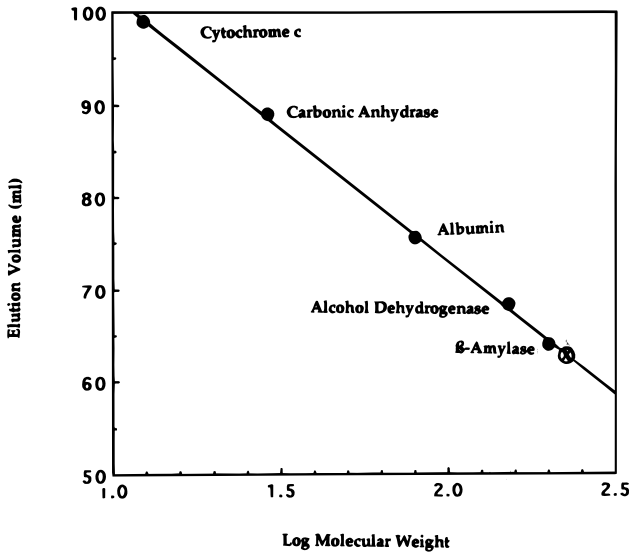

FIGURE 3: Molecular weight of native β-endorphin endopeptidase. β-Endorphin endopeptidase and protein standards were chromatographed on Superdex 200. The location of the endopeptidase on the standard curve is shown by the crossed circle (⊗). The molecular masses of the proteins are in kDa.

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          (86)                                                (109)
Rat IDE   S S A A L D V H I G S L S D P P N I P G L S N F
EL4 GEGE  S S A ? L D V H I G S L S D P ? N I P G L S ? F

          (714)                                               (734)
Rat IDE   A F I P Q L L S R L H I E A L L H G N I T
EL4 GEGE  A F I P Q L L S R L H I E A L L H G N I T

          (907)                                 (920)
Rat IDE   Y W G E I I S Q Q Y N Y D R
EL4 GEGE  Y W G E I I S Q Q Y N Y D R
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FIGURE 4: Sequence comparison of β-endorphin endopeptidase with rat insulin-degrading enzyme. The sequence for the β-endorphin endopeptidase was obtained as described in Materials and Methods. Rat insulin-degrading enzyme sequence was obtained from Baumeister et al. (1993).

Identification of the β-Endorphin Endopeptidase As γ-Endorphin-Generating Enzyme. In order to determine whether the secreted β-endorphin-cleaving activity is in fact γ-endorphin-generating enzyme, we used an antisera raised against the EL-4 peptidase to test for cross-reactivity with rat testis γ-endorphin-generating enzyme. This antibody cross-reacted with the protein of the same molecular weight from rat testis, Figure 6. Partial purification of the rat testis γ-endorphin-generating enzyme according to the procedure of Lebouille et al. (1989), confirmed that this enzyme is in fact β-endorphin-cleaving enzyme and insulin-degrading enzyme, since these activities were localized to the same fractions. The γ-endorphin-generating activity from testis and the secreted and intracellular β-endorphin-cleaving activity from EL-4 cells eluted as a 220 kDa protein on a calibrated gel-filtration column.

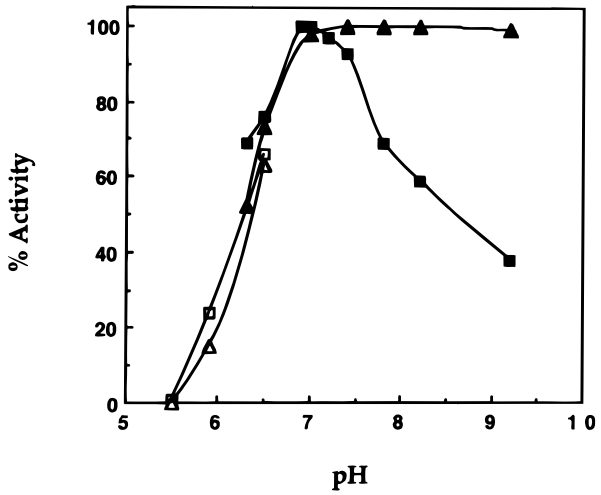

FIGURE 5: pH dependence of endopeptidase activity. β-Endorphin cleavage activity was determined as described in Materials and Methods at the indicated pH using 100 mM 2-[*N*-morpholino]-ethanesulfonic acid (MES) (open symbols) or 100 mM 1,3-bis-[tris(hydroxymethyl)methylamino]propane (Bis-Tris-propane) (closed symbols). Activity is expressed relative to the maximal activity obtained. β-Endorphin-cleaving activity (■) is based on formation of β-endorphin 1−17 and 1−18. Dynorphin B 1−13 activity (▲) is based on the formation of dynorphin B 1−6.

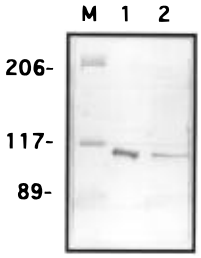

FIGURE 6: Immunoblotting of testis γ-endorphin-generating enzyme. (Lane 1) 0.25 μg of purified IDE from EL-4 cells. (Lane 2) 50 μg of partially purified γ-endorphin-generating enzyme from rat testis. The protein samples were separated by a 10% SDS−PAGE, transferred to PVDF, and developed as described in Materials and Methods.

Substrate Specificity and Kinetic Parameters. Since the results described above demonstrate that β-endorphin is a previously unidentified substrate for insulin-degrading enzyme, we examined a number of other peptides as potential substrates for the enzyme. In these studies the K_m for each peptide was determined by using it as an alternative substrate inhibitor. V_{max} was determined by measuring the rate of peptide hydrolysis at a saturating substrate concentration by following its disappearance using reverse-phase HPLC. By following disappearance of the substrate, cleavage at any single bond would be detected. The peptide cleavage site(s) was determined by collecting product peaks and determining their N-terminal sequence by automated Edman degradation. As shown in Table 2, the enzyme hydrolyzed the 29 amino acid peptide GRF, the 31 amino acid peptide β-endorphin, and the 17 amino acid peptide dynorphin A

Table 2: Kinetic Constants for the Hydrolysis of Various Peptides by EL4-IDE[a]

peptide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
insulin B chain	7.8 ± 1.4[b]	0.47 ± 0.04	16.6
insulin (porcine)	0.56 ± 0.01[b]	0.13 ± 0.02	4.30
GRF (1−29)	23.4 ± 2.8[b]	11.9 ± 1.9	1.97
β-endorphin (1−31)	20.7 ± 2.0[c]	13.0 ± 1.0	1.59
dynorphin B (1−13)	15.07 ± 0.17	18.3 ± 2.1	0.83
dynorphin A (1−17)	18.4 ± 1.2	37.4 ± 3.5	0.49
dynorphin B (1−9)	10.34 ± 0.44	26.8 ± 2.5	0.38
pancreastatin (1−49)	10.6 ± 2.0	41.7 ± 7.5	0.25
dynorphin A (1−13)	3.74 ± 0.04	40.6 ± 3.8	0.09
dynorphin A (1−10)	3.74 ± 0.04	39.4 ± 0.4	0.09
dynorphin A (1−8)	0.66 ± 0.02	63.5 ± 7.5	0.01
dynorphin A (1−9)	0.33 ± 0.01	60.5 ± 5.8	>0.01

[a] Purified enzyme (0.1−3 μg) was incubated at 37 °C in 80 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl for 0−6 h, with the indicated substrate at a concentration of 0.1 mM in a 100 μL reaction mixture. At different time intervals aliquots were taken and analyzed for substrate disappearance by reverse-phase HPLC. The linear portion of each reaction was used to determine the rate of hydrolysis. The following peptides were not cleaved at a detectable rate: dynorphin A (1−5), Y-G-G-F-L; dynorphin A (1−7), Y-G-G-F-L-R-R; substance P (1−6), R-P-K-P-Q-Q; substance P (1−7), R-P-K-P-Q-Q-F; leucopyrokinin fragment, F-T-P-R-L; α-neoendorphin, Y-G-G-F-L-R-K-Y-P-K; β-neoendorphin, Y-G-G-F-L-R-K-Y-P; bradykinin, R-P-P-G-F-S-P-F-S; angiotensin I (1−10), D-R-V-Y-I-H-P-F-H-L; β-endorphin (1-17), Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L; β-endorphin (18−31), F-K-N-A-I-I-K-N-A-Y-K-K-G-E; ACTH (1−24), S-Y-S-M-E-H-F-R-W-G-K-P-V-G-K-K-R-R-P-V-K-V-Y-P; galanin (rat), G-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-I-D-N-H-R-S-F-S-D-K-H-G-L-T. [b] These substrates were cleaved at more than one site, therefore the reported k_{cat} is an apparent rate constant representing an average for all rapidly cleaved sites. [c] β-Endorphin is cleaved at two positions, Leu^{17}-Phe^{18} and Phe^{18}-Lys^{19}, at equal rates; therefore the k_{cat} per site is 10.3 min^{-1}.

with approximately the same k_{cat}. The 31 amino acid insulin B chain and the 49 amino acid peptide pancreastatin were cleaved at $\sim^1/_2$ the k_{cat} of β-endorphin, while the 13 amino acid peptide, dynorphin B-13 was cleaved at $\sim^3/_4$ the k_{cat} of β-endorphin. Interestingly, insulin, the substrate for which the enzyme gets its name, exhibited a k_{cat} value 1/40 that of β-endorphin.

Galanin which is the same size as β-endorphin was not cleaved at a detectable rate. In addition, the enzyme did not cleave ACTH 1−24, a series of dynorphin A related peptides smaller than 8 amino acids, β-endorphin 1−17 (γ-endorphin), and other peptides listed in the legend of Table 2. Dynorphin B related peptides exhibited k_{cat} values at least 8-fold greater than dynorphin A related peptides. A comparison of dynorphin B 1−9 and 1−13 to dynorphin A 1−9 and 1−13 indicates that the residues in P′ sites are of importance. Comparison of k_{cat} values within the dynorphin series indicates a preference of the enzyme for extended substrates.

As indicated in Table 3, β-endorphin was cleaved at both the Leu^{17}-Phe^{18} and the Phe^{18}-Lys^{19} bonds. When the rate of formation of β-endorphin 1−17 was compared to that of β-endorphin 1−18, both products were formed at equal rates, Figure 7. Dynorphin B-13 was cleaved between Arg^6-Arg^7, and dynorphin A-17 was cleaved between Lys^{11}-Leu^{12}, while pancreastatin was cleaved primarily between Gly^1-Trp^2. Insulin B chain was cleaved most rapidly between Glu^{13}-Ala^{14}, with a slower secondary cleavage at Tyr^{16}-Leu^{17}. GRF is cleaved at four positions; between Ser^9-Tyr^{10}, Arg^{11}-Lys^{12}, Ser^{18}-Ala^{19}, and Arg^{20}-Lys^{21}. The latter cleavage was a minor one. Although the cleavage pattern of pancreastatin is what might be expected for an aminopeptidase, this reaction was not affected by added bestatin, a specific aminopeptidase inhibitor, nor could we detect aminopeptidase activity in the enzyme preparation using alanine β-naphthylamide as substrate.

To ensure the involvement of only one enzyme in the cleavage of both hydrophobic and basic residues, the extent of enzyme inactivation by heat and cystine was determined with respect to activity toward pancreastatin (hydrophobic cleavage), β-endorphin (basic and hydrophobic cleavage) and dynorphin B 1−13 (basic cleavage). Previously, cystine was found to reversibly inactivate the enzyme (Safavi and Hersh, unpublished results). All the activities were lost at the same rate, thus providing strong evidence that all of the reported cleavages are due to a single enzyme (data not shown). Furthermore, the same extent of reactivation of the cystine-inactivated enzyme by β-mercaptoethanol was obtained when measured with either β-endorphin or dynorphin B 1−13.

K_m values for various peptides were obtained by using them as alternative substrate inhibitors of β-endorphin hydrolysis. Insulin had the highest affinity for the enzyme, K_m of 130 nM (Figure 8, bottom), followed by insulin B chain with a 3.5-fold higher K_m. β-Endorphin (Figure 8, top) and GRF had K_m values of $\sim$13 μM. The enzyme showed a higher affinity for dynorphin B peptides than dynorphin A peptides by at least 2-fold. Dynorphin A peptides of fewer than 10 amino acids had K_m values greater than 60 μM. However, there was no direct relationship between size of the peptide and K_m. This is apparent when the insulin B chain (30 amino acids, $K_m = 0.47$ μM) is compared with pancreastatin (49 amino acids, $K_m = 41.7$ μM) and dynorphin A 1−8 (8 amino acids, $K_m = 63.5$ μM).

As shown in Table 2, GRF and β-endorphin have k_{cat}/K_m values which are within 2−3-fold of insulin, while dynorphin B 1−13, dynorphin A 1−17, dynorphin B 1−9, and pancreastatin exhibit k_{cat}/K_m values 1/5 to 1/20 that of insulin.

DISCUSSION

We have purified from the EL-4 thymoma cell line a secreted endopeptidase that cleaves β-endorphin to β-endorphin 1−17 (γ-endorphin), β-endorphin 1−18, and their C-terminal fragments. This activity corresponds to the previously described γ-endorphin-generating activity and is in fact an activity of insulin-degrading enzyme. In addition to EL-4 cells, we have observed the same enzyme activity being secreted by Jurkat T cells (data not shown) and primary cultures of anti-CD3-activated murine $CD4^+$ T cells (Miller et., 1995).

The native molecular mass of insulin-degrading enzyme from different tissues has been reported to vary between 100 and 300 kDa, making it unclear whether the enzyme exists as a monomer, dimer, or trimer (Duckworth, 1995). We found the native molecular mass of both endogenous and secreted forms of insulin-degrading enzyme to be $\sim$220 kDa, making it a dimer.

We have shown that insulin-degrading enzyme has a broad substrate specificity. The enzyme cleaves the physiological peptides β-endorphin, GRF, dynorphin A 1−17, dynorphin B 1−13, and pancreastatin all with k_{cat} values at least 20 times greater than insulin but all with higher K_m values. Thus these substrates become more nearly equivalent in terms of

Table 3: Cleavage Sites of Various Peptides by EL4-IDE[a]

peptide	peptide sequence and bond cleavages
dynorphin A (1−8)	Y-G-G-F-L-**R**-**R**-I
dynorphin A (1−9)	Y-G-G-F-L-**R**-**R**-I-R
dynorphin B (1−9)	Y-G-G-F-L-**R**-**R**-Q-F
dynorphin A (1−10)	Y-G-G-F-L-**R**-**R**-I-R-P
dynorphin A (1−13)	Y-G-G-F-L-**R**-**R**-I-R-P-K-L-K
dynorphin B (1−13)	Y-G-G-F-L-**R**-**R**-Q-F-L-V-V-T
dynorphin A (1−17)	Y-G-G-F-L-R-R-I-R-P-**K**-**L**-K-W-D-N-E
β-endorphin (1−31)	Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-**L**-**F**-**K**-N-A-I-I-K-N-A-Y-K-K-G-E
insulin B chain	F-V-N-Q-H-L-C [SO_3H] -G-S-H-L-V-**E**-**A**-L-Y*L-V-C-[SO_3H] -G-E-R-G-F-F-Y-Y-P-K-A
GRF (1−29)	Y-A-D-A-I-F-T-N-**S**-**Y**-**R**-**K**-V-L-G-G-L-**S**-**A**-R*K-L-L-Q-D-I-NIe-S-R
pancreastatin (1−49)	**G**-**W**-P-Q-A-P-A-M-D-G-A-G-K-T-G-A-E-E-A-Q-P-P-E-G-K-G-A- R-E-H-S-R-Q-E-E-E-E-E-T-A-G-A-P-Q-G-L-F-R-G

[a] Purified enzyme (0.1−3 μg) was incubated at 37 °C in 80 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, with the indicated substrate at a concentration of 0.1 mM in a 100−200 μL reaction mixture. After 3−6 h the reactions were stopped by addition of 10 μL of 0.5% TFA. Sites of cleavage were determined by collecting the products by HPLC and subjecting them to N-terminal sequence analysis. The major cleavage sites are represented by **bold letters**, and the minor cleavage sites are represented by *.

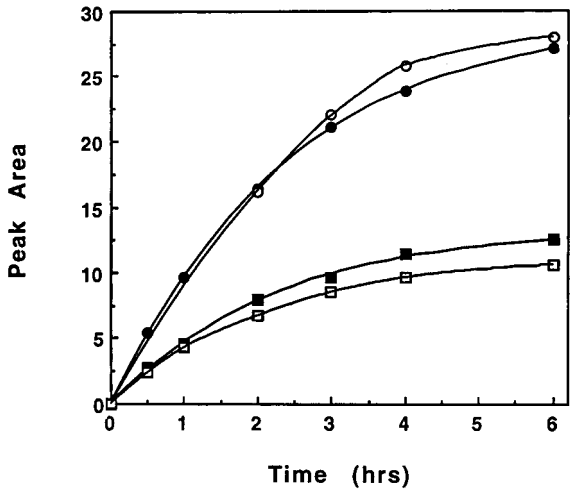

FIGURE 7: Stoichiometry of β-endorphin metabolite formation. The reaction of purified enzyme (0.1 μg) with 100 μM β-endorphin was measured at various times up to 6 h at 37 °C in 80 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl. The rate of product formation was determined from the peak area of HPLC chromatograms, with products identified by N-terminal sequencing. (●) β-Endorphin 1−17, (○) β-endorphin 1−18, (■) β-endorphin 18−31, and (□) β-endorphin 19−31.

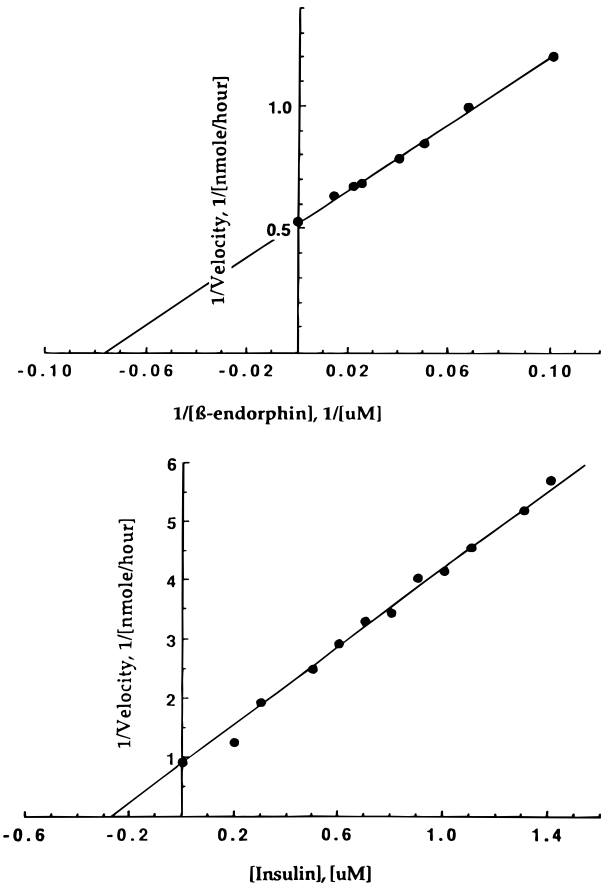

FIGURE 8: K_m of insulin-degrading enzyme for insulin and β-endorphin. K_m values were obtained as described in Materials and Methods. (Top) Reciprocal plot showing 1/velocity vs 1/[β-endorphin]. (Bottom) Plot of 1/velocity vs [insulin] at a fixed concentration of β-endorphin (13 μM).

their specificity constant, k_{cat}/K_m. The 10−50 μM K_m values for β-endorphin, GRF, pancreastatin, and dynorphins do not preclude these peptides from being physiological substrates. Leu- and met-enkephalin, which are known physiological substrates for neprilysin (Roques et al., 1980), exhibit kinetic K_m values of 47 and 13 μM, respectively (Hersh & Morihara, 1986). In addition, although the K_m for β-endorphin as well as the dynorphin peptides appears high compared to insulin, the local peptide concentration at the site of peptide release could be relatively high and approach or exceed the observed K_m. In addition to the substrates presented here, the degradation of other peptides by IDE has been reported. Iodinated glucagon is cleaved at $^4/_5$ the rate of iodinated insulin (Shii et al., 1986), iodinated insulin growth factor I and II are cleaved by IDE at a rate of 3% and 18% of iodinated insulin, respectively (Roth et al., 1984), and iodinated transforming growth factor-α is cleaved at the same rate as iodinated insulin. However, iodinated epidermal growth factor is not cleaved at a detectable rate (Gehm et al., 1991). Proinsulin which acts as a competitive inhibitor is cleaved ∼100 times more slowly than insulin by IDE (Duckworth, 1988). Thus there is ample evidence in the literature that IDE is active toward a number of peptides other than insulin. However, the kinetic parameters for these substrates have not been fully determined, and thus their potential as physiological substrates is difficult to evaluate.

The substrate specificity for IDE observed in this study is one directed at the amino side of either hydrophobic or basic residues. We have used inactivation of the enzyme by heat and cystine to demonstrate that cleavage at both hydrophobic and basic residues is catalyzed by the same enzyme. However, not all basic and hydrophobic amino acids are recognized. Using a series of dynorphin-related peptides it has been shown that a P_2' residue is required for activity. This specificity is consistent with the previously reported

cleavage sites in other peptides. For insulin, cleavage in the A chain occurs between Leu^{13}-Tyr^{14} and Tyr^{14}-Gln^{15} while cleavage in the B chain occurs between His^{10}-Leu^{11}, Ala^{14}-Leu^{15}, Tyr^{16}-Leu^{17} and Phe^{25}-Tyr^{26} (Duckworth et al., 1988). Atrial natriuretic peptide is cleaved between Ser^{25}-Phe^{26}, Arg^{3}-Arg^{4}, Cys^{7}-Phe^{8}, and Asp^{13}-Arg^{14} bonds, brain natriuretic peptide is cleaved between Gly^{6}-Arg^{7}, Asp^{10}-Arg^{11}, and Arg^{24}-Arg^{25} bonds, C-type natriuretic peptide is cleaved between Gly^{8}-Leu^{9} and Asp^{12}-Arg^{13} (Muller et al., 1992), and glucagon is cleaved between Arg^{17}-Arg^{18} (Rose et al., 1988). All of these sites are found to be at the amino side of basic or hydrophobic residues.

The physiological function of insulin-degrading enzyme remains to be firmly established. Its role in degrading insulin internalized with the insulin receptor has recently been questioned (Authier et al., 1994). The localization of the enzyme to the cytosol and peroxisomes would seem to preclude this function. The presence of the enzyme in T-cells might suggest a role for the enzyme in modulating β-endorphin action on lymphocytes and for the conversion of β-endorphin to its active metabolites γ-endorphin and β-endorphin 18−31.

In addition to this study, several other reports have provided information which suggests other possible physiological roles for IDE. Semple et al. (1989) have shown that a peptide derived from the degradation of insulin by IDE is immunogenic and implies a role of the enzyme in antigen processing. However, the potential role of IDE in antigen processing has not been extended beyond insulin. Fagan et al. (1991) have shown the possible involvement of IDE in degrading oxidatively damaged haemoglobin. Duckworth et al. (1994) provided evidence of an interaction between IDE and the multicatalytic proteinase and the inhibitory effect of IDE on the multicatalytic proteinase when bound with insulin. Kupfer et al. (1991) proposed a possible interaction between IDE and steroid receptors by demonstrating that IDE enhances DNA binding of these receptors.

It has been observed that testis contain a high level of insulin-degrading enzyme/γ-endorphin-generating enzyme and that its expression is developmentally regulated in this tissue (Lebouille et al., 1986; Baumeister et al., 1993). Lebouille et al. (1986) have found that the highest level of this enzyme is in the tubuli and germinal cells in testis. These cells are in close proximity to the Leydig cells which have been shown to synthesize POMC and contain β-endorphin (Pintar et al., 1984). Therefore, within testis, there are cells which could make and secrete β-endorphin and, in close proximity, cells that can secrete an enzyme that is capable of cleaving β-endorphin. These findings suggest a possible role for insulin-degrading enzyme/γ-endorphin-generating enzyme in spermatogenesis.

Recently Authier et al. (1995) showed that IDE could cleave a synthetic peptide corresponding to the leader peptide of the peroxisomal enzyme thiolase. On the basis of this observation they suggested that the enzyme might either process thiolase in the peroxisome or degrade the released leader peptide following processing of thiolase by another protease. Our results favor the latter role. We as well as Authier et al. have not been able to demonstrate cleavage of a protein, only peptides, by IDE.

In summary we have identified and purified insulin-degrading enzyme from EL-4 cells. We have shown that insulin-degrading enzyme cleaves β-endorphin as well as a number of other biologically active peptides. These results suggest that like other peptidases such as neprilysin, aminopeptidase M, and endopeptidase 24.15, insulin-degrading enzyme might regulate the action of a number of peptides and thus the physiological processes controlled by these peptides.

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