Characterization of a Nonchymotrypsin-like Endopeptidase from Anterior Pituitary That Hydrolyzes Luteinizing Hormone–Releasing Hormone at the Tyrosyl–Glycine and Histidyl–Tryptophan Bonds†

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ABSTRACT: A neutral endopeptidase which degrades luteinizing hormone–releasing hormone (LH–RH, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) has been purified 900-fold from extracts of bovine anterior pituitary. This Ca^{2+}-independent enzyme of 83 000 molecular weight (as estimated by gel filtration) cleaves LH–RH (K_M = 180 μM) at the Tyr^5–Gly^6 and His^2–Trp^3 bonds. Its activity is inhibited by the SH-reactive agents *N*-ethylmaleimide and *p*-(chloromercuri)benzoate but not by the OH-reactive agent diisopropyl fluorophosphate. Hydrolysis of the fluorogenic chymotrypsin substrate glutaryl-Gly-Gly-Phe-β-naphthylamide by this endopeptidase could not be detected. These properties differentiate the endopeptidase from chymotrypsin and from a glutaryl-Gly-Gly-Phe-β-naphthylamide hydrolyzing activity of high molecular weight, which has been isolated from the same tissue and also hydrolyzes internal bonds of LH–RH.

The neuropeptide luteinizing hormone–releasing hormone (LH–RH, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)[1] participates in synaptic events and the hypothalamic control of adenohypophyseal hormone secretion [for a review see Guillemin (1978) and Schally (1978)]. It is rapidly inactivated by pancreatic chymotrypsin (Schally et al., 1971) and by enzymes present in other tissues [for a review see Marks (1978) and Griffiths & Kelly (1979)]. Alterations in the rate of its degradation are possibly involved in the regulation of its activity (Griffiths et al., 1976). From brain and pituitary tissue, a pyroglutamate aminopeptidase (Bauer et al., 1979) and a post-proline-cleaving enzyme (Knisatschek & Bauer, 1979; Hersh & McKelvy, 1979) have been isolated, which cleave the decapeptide at the $<Glu^1$–His^2 bond and the Pro^9–Gly-$NH_2{}^{10}$ bond. Cleavage of the Tyr^5–Gly^6 bond (Hudson et al., 1976) and/or the Gly^6–Leu^7 bond (Akopyan et al., 1979; Koch et al., 1974; Fridkin et al., 1977) by brain, pituitary, and liver extracts has been reported, but the determination of these two cleavage sites has been controversal. Here we report on the purification and characterization of two peptidases cleaving LH–RH at internal sites.

Experimental Procedure

Materials

Whole bovine pituitaries were purchased from Pel Freeze. Chromatography papers and DE-52-cellulose were products from Whatman. Ultrogel AcA 44 was obtained from LKB, phenyl-Sepharose CL-4B was from Pharmacia Fine Chemicals, and hypatite C was from Clarkson Chemical Co. For scintillation counting, emulsifier scintillator 299 from Packard was used. [*pyroglutamyl*-3,4-^{3}H]LH–RH (40 Ci/mmol) was purchased from New England Nuclear. LH–RH, glutaryl-Gly-Gly-Phe-2-NNap, Leu-2-NNap, Ser-Tyr-2-NNap, Boc-Gly-Phe, Boc-Gly-ONSu, and Boc-Phe were products from Bachem AG, Bubendorf, Switzerland. <Glu-His-Trp and Gly-Leu-Arg-Pro-Gly-NH_2 were obtained from UCB, Belgium. Succinic acid monomethyl ester was purchased from EGA Chemie, West Germany. Bovine α-chymotrypsin (catalog no. 2308) was obtained from Merck AG, West Germany; glucose-6-phosphate dehydrogenase, alkaline phosphatase, and malate dehydrogenase were from Boehringer, Mannheim, West Germany.

Methods

The following buffers were used in this study: buffer A, 1 mM potassium phosphate, pH 7.4, and 1 mM dithioerythritol; buffer B, 10 mM potassium phosphate, pH 7.4, and 1 mM dithioerythritol; buffer C, 20 mM potassium phosphate, pH 7.4, and 1 mM dithioerythritol; buffer D, 50 mM potassium phosphate, pH 7.4, and 1 mM dithioerythritol; buffer E, 2% formic acid and 8% acetic acid, pH 1.9.

Protein Determination. Protein was determined with Coomassie Blue according to Spector (1978) with bovine serum albumin as the standard.

Purification of [^{3}*H*]*LH–RH.* [^{3}H]LH–RH (220 μCi) was diluted with 45 nmol of unlabeled LH–RH and subjected to ion-exchange chromatography on a CM-cellulose column (0.8 × 2 cm) equilibrated with 10 mM ammonium acetate buffer, pH 4.6. After washing with 5 mL of the same buffer, a gradient established from 20 mL of 10 mM ammonium acetate buffer, pH 4.6, and 20 mL of 100 mM ammonium acetate buffer, pH 7.0, was applied (flow rate 12 mL/h). The fractions (0.5 mL) were counted for radioactivity, and the LH–RH-containing fractions (0.9–1.1 mS) were pooled.

Preparation of [^{3}*H*]*<Glu-His-Trp and* [^{3}*H*]*<Glu-His-Trp-Ser-Tyr.* Purified [^{3}H]LH–RH (25 μCi) was diluted with 300 nmol of unlabeled LH–RH and incubated with 5 ng of α-chymotrypsin in 50 mM Tris-HCl buffer, pH 8.0, and 10 mM $CaCl_2$ (0.5-mL final volume) at 37 °C for 30 min. The reaction was stopped by adding 2 volumes of ice-cold methanol. After standing for 30 min at 0 °C, the reaction mixture was centrifuged for 15 min at 9000*g*. The supernatant was concentrated in vacuo and applied to Whatman No. 3 paper. The synthetic marker peptides <Glu-His-Trp and <Glu-His-Trp-Ser-Tyr were spotted at the edge of the paper. High-voltage electrophoresis (60 V/cm) at pH 1.9 (buffer E) was terminated, when migration of 11 cm was observed for the colored

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[1] The abbreviations used follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature. All optically active amino acids are of L configuration. Additional abbreviations used: <Glu, pyroglutamic acid; Suc, succinyl; Cbz, benzyloxycarbonyl; Boc, butoxycarbonyl, ONSu, succinimidooxy; 2-NNap, β-naphthylamide; CM, carboxymethyl; mS, mSiemens.

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marker phenosafranine. After the paper was dried and scanned for radioactivity, the outer border of the sheet was cut off and sprayed with Pauly's reagent. The radioactive material from the segments corresponding to the position of the markers was eluted with 200 μL of water.

Synthesis of Peptide Derivatives. Using standard procedures of peptide synthesis in solution (Houben & Weyl, 1974a,b), the following peptide derivatives were synthesized.

N-Suc-Phe-Gly-Leu-2-NNap was prepared as follows. Boc-Gly-ONSu was reacted with Leu-2-NNap in the presence of triethylamine. The Boc group was removed by 2 N HBr in acetic acid, and the resulting Gly-Leu-2-NNap was coupled with Boc-Phe by the *N*,*N*′-dicyclohexylcarbodiimide–1-hydroxybenzotriazole method. After removing the Boc group by trifluoroacetic acid, Phe-Gly-Leu-2-NNap was acylated with succinic anhydride (mp 169–171 °C).

N-Suc-Tyr-Gly-Leu-2-NNap was synthesized in the following steps. Succinic acid monomethyl ester was coupled with *N*-hydroxysuccinimide by using *N*,*N*′-dicyclohexylcarbodiimide and then reacted with tyrosine. The product was coupled with Gly-Leu-2-NNap (prepared as described above) by the *N*,*N*′-dicyclohexylcarbodiimide–1-hydroxybenzotriazole procedure, and the methyl ester was saponified with potassium hydroxide (mp 165–167 °C).

N-Cbz-Ser-Tyr-2-NNap was prepared by reacting Ser-Tyr-2-NNap with chloroformic acid benzyl ester (mp 196–198 °C).

Glutaryl-Gly-Gly-Phe-2-NNap was purified by thin-layer chromatography on silica gel plates using the following solvent systems: $CHCl_3$–CH_3OH–NH_3 aqueous (90:10:1 v/v/v), R_f = 0.05; $CHCl_3$–CH_3OH–CH_3COOH (90:10:3 v/v/v), R_f = 0.27. The material was eluted from the silica gel with methanol.

Tissue Extraction and Ammonium Sulfate Fractionation. All steps were carried out at 4 °C. Centrifugation was always performed at 9000*g* for 15 min.

Bovine anterior pituitaries (100 g wet weight) were minced with scissors, washed 3 times with buffer D, and homogenized in 800 mL of buffer D under nitrogen atmosphere by using an Omni-Mix (three 1-min periods at maximal speed). After centrifugation the supernatant (765 mL) was aspirated, and saturated ammonium sulfate solution in buffer D was slowly added under constant stirring (35% saturation). After being stirred for an additional 30 min, the precipitate was removed by centrifugation. To the supernatant (1130 mL) saturated ammonium sulfate solution in buffer D was slowly added under constant stirring (65% saturation). After being stirred for 30 min, the precipitated material was collected by centrifugation. The pellet was dissolved in 90 mL of buffer C and dialyzed against four 1-L batches of buffer C for a total of 24 h. Insoluble material was removed by centrifugation.

Ion-Exchange Chromatography on DEAE-cellulose. The solution (100 mL) was applied to a DEAE-cellulose column (4.5 × 7 cm) equilibrated with buffer C. The column was washed with 350 mL of buffer C. Then a linear KCl gradient (0–100 mM KCl in 700 mL of buffer C) was applied, followed by a second KCl gradient (100–350 mM KCl in 500 mL of buffer C). Fractions of 7 mL were collected at a flow rate of 75 mL/h. The fractions containing the LH–RH degrading endopeptidase and the fractions containing the glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity were pooled separately. After ammonium sulfate precipitation (70% saturation), the preparations containing the LH–RH degrading endopeptidase and the glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity were dissolved in 3.4 and 10 mL of buffer D and dialyzed against the same buffer for a total of 12 h.

Dialysis against Water. The LH–RH degrading endopeptidase was dialyzed at 1 °C against six 1-L batches of water containing 1 mM dithioerythritol for 72 h. The precipitated protein was removed by centrifugation. The supernatant (5.4 mL) was supplemented with 280 μL of 1 M potassium phosphate buffer, pH 7.4, yielding 50 mM final buffer concentration.

Gel Filtration through Ultrogel AcA 44. The enzyme preparations (2.6 and 4 mL) were subjected to gel filtration through Ultrogel AcA 44 columns (2.5 × 100 cm) equilibrated with buffer D. The columns were eluted with buffer D at a flow rate of 25 mL/h, and fractions of 3.5 mL were collected. The fractions containing the LH–RH degrading endopeptidase were pooled and concentrated to 2 mL by ultrafiltration through an Amicon UM 10 membrane at 3 bar.

Hydrophobic Interaction Chromatography on Phenyl-Sepharose CL-4B. One milliliter of the LH–RH degrading endopeptidase preparation was mixed with saturated ammonium sulfate solution (20% saturation) and applied to a phenyl-Sepharose CL-4B column (1 × 2 cm) equilibrated with buffer B containing ammonium sulfate (20% saturation). The column was washed with 20 mL of equilibration buffer. Then a linear ammonium sulfate gradient (20–0% saturation; 200 mL of buffer B) was applied, and fractions of 3 mL were collected at a flow rate of 30 mL/h. The active fractions were pooled (55 mL) and dialyzed for 12 h against two 1-L batches of buffer A.

Adsorption Chromatography on Hypatite C. The dialysate (55 mL) was applied to a Hypatite C column (1 × 1.5 cm) equilibrated with buffer A. The column was washed with 20 mL of buffer A and then eluted with 100 mL of a linear gradient (1–75 mM potassiium phosphate buffer, pH 7.4, and 1 mM dithioerythritol). Fractions of 2 mL were collected at a flow rate of 30 mL/h. The fractions containing the enzyme activity were pooled and concentrated by dialysis against 1 L of 20% poly(ethylene glycol) 6000 in buffer D, yielding 3.4 mL of enzyme solution.

Polyacrylamide Gel Electrophoresis. The LH–RH degrading endopeptidase (50 μL) in 10% sucrose was applied on a 6% polyacrylamide gel prerun at 6 mA/gel in 100 mM potassium phosphate buffer, pH 8.0, and 1 mM dithioerythritol. Electrophoresis was carried out in the same buffer for 1 h at 3 mA/gel and then for 7 h at 6 mA/gel. The gel was cut into slices (2 mm), which were homogenized in 250 μL of buffer D and kept at 4 °C over night. The polyacrylamide was removed by centrifugation, and the supernatant was assayed for enzyme activity.

Enzyme Assays. (1) Enzyme Tests with Radioactively Labeled Substrates. Unless otherwise stated, 10 μL of enzyme preparation, 15 μL of buffer D containing 0.2 mM EDTA, and 5 μL of the substrate solutions were mixed and incubated at 37 °C.

(a) Degradation of [^3H]LH–RH. The final concentration of LH–RH in the assay was 2 μM. At given time intervals, aliquots (5 μL) of the reaction mixture were withdrawn and spotted on P 81 cellulose phosphate paper. After development in 25 mM ammonium acetate (ascending, front 17 cm), the sheets were dried. Under these conditions only the strongly basic LH–RH remains at the origin, whereas the less basic and acidic N-terminal fragments are eluted. The segments at the origin (1.5 × 2 cm) were cut off, placed in scintillation vials, and eluted with 1 mL of 2 N ammonia solution. Ten minutes later 9 mL of scintillation cocktail was added before scintillation counting.

(*b*) *Degradation of* [^3H]<*Glu-His-Trp-Ser-Tyr*. The final concentration of <Glu-His-Trp-Ser-Tyr in the assay was 50 μM. Aliquots (2 μL) of the reaction mixture were spotted on CM-cellulose paper. After development in water (ascending, front 10 cm), the strip was segmented, and the radioactivity in the segments containing <Glu-His-Trp-Ser-Tyr (R_f = 0.45) and <Glu-His (R_f = 0.78) was determined by scintillation counting as described above.

(*c*) *Degradation of* [^3H]<*Glu-His-Trp*. The final concentration of <Glu-His-Trp in the assay was 50 μM. Aliquots (2 μL) of the reaction mixture were resolved by high-voltage electrophoresis (60 V/cm) at pH 1.9 (buffer E) on Whatman No. 3 paper. <Glu-His-Trp and <Glu-His were used as markers. Electrophoresis was terminated when migration of 11 cm was observed for phenosafranine. After being dried, the sheet was scanned for radioactivity.

(*2*) *Enzyme Tests with Fluorogenic Substrates*. The incubation volume was always 3 mL. Unless otherwise stated, the enzymatic liberation of β-naphthylamine from the substrates at 37 °C was determined fluorometrically by using a Perkin-Elmer Fluorospectrophotometer MPF 44 (excitation at 340 nm; emission at 410 nm).

(*a*) *Glutaryl-Gly-Gly-Phe-2-NNap Hydrolyzing Enzyme and Chymotrypsin*. The glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity (200 μL of the fractions) was assayed with 0.1 mM substrate in 50 mM potassium phosphate buffer, pH 7.4 (incubation time 2 h). Chymotrypsin (3.8 μg) was incubated with the same substrate in 50 mM Tris-HCl buffer, pH 8.0, for 10 min.

(*b*) *Aminopeptidases*. Aliquots of the fractions (50 μL) were incubated with 0.1 mM Leu-2-NNap in 50 mM potassium phosphate buffer, pH 7.4, and 1 mM $MgCl_2$ for 30 min.

(*c*) *Pyroglutamate Aminopeptidase*. Aliquots of the fractions (50 μL) were incubated with 0.1 mM <Glu-2-NNap in buffer D and 2 mM EDTA for 60 min.

(*d*) *Post-Proline-Cleaving Enzyme*. Aliquots of the fractions (100 μL) were incubated with 0.01 mM Cbz-Gly-Pro-2-NNap in 100 mM potassium phosphate buffer, pH 7.4, 1 mM dithioerythritol and 1 mM EDTA for 15 min (Knisatschek et al., 1980).

Carboxypeptidases were assayed by using Boc-Gly-Phe as described (Bergmeyer, 1970).

Amino Acid Analysis. Peptides were hydrolyzed in vacuo for 22 h at 110 °C in 6 N HCl containing 2 mg of phenol per mL. Amino acid analysis was carried out on a Durrum D-500 analyzer using the standard program of the "one-column" method.

Results

Enzyme Purification. Inactivation of LH-RH by pituitary extracts at neutral pH is mainly due to enzymatic cleavage of the decapeptide amide at the <Glu^1–His^2 bond, the Pro^9–Gly-$NH_2{}^{10}$ bond, and internal peptide bonds (Bauer et al., 1979). The activity of the pyroglutamate aminopeptidase and the post-proline-cleaving enzyme can be inhibited completely by 2-iodoacetamide and diisopropyl fluorophosphate (Knisatschek & Bauer, 1979; unpublished observations). The endopeptidase which catalyzes the hydrolysis of internal peptide bonds and which is described in this study is unaffected by these inhibitors (see below). Therefore, in crude extracts the activity of this endopeptidase can be determined selectively in the presence of 2-iodoacetamide and diisopropyl fluorophosphate.

By ion-exchange chromatography on DEAE-cellulose, two fractions with LH-RH degrading activity are obtained (see Figure 1). The major fraction elutes in the range of 2.0–3.7 mS and contains the pyroglutamate aminopeptidase and the post-proline-cleaving enzyme. The minor activity, which is recovered in the range of 1.2–1.7 mS, is the LH-RH degrading endopeptidase. Hydrolysis of the fluorogenic chymotrypsin substrate glutaryl-Gly-Gly-Phe-2-NNap is catalyzed by an enzymatic activity, which elutes in the range of 4–5 mS.

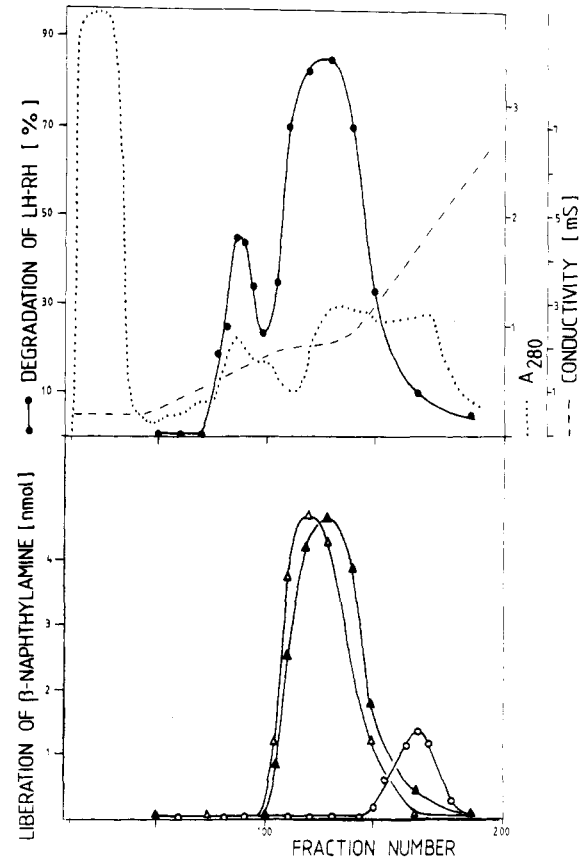

FIGURE 1: Ion-exchange chromatography on DEAE-cellulose. The material of the anterior pituitary tissue extracts precipitating at 35–65% ammonium sulfate saturation was subjected to ion-exchange chromatography on DEAE-cellulose. KCl was used for gradient elution. The degradation of LH-RH (●) and the hydrolysis of glutaryl-Gly-Gly-Phe-2-NNap (○), <Glu-2-NNap (△), and Cbz-Gly-Pro-2-NNap (▲) were determined as described under Methods.

The elution behavior of the glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity and the LH-RH degrading endopeptidase on Ultrogel AcA 44 is shown in Figure 2. The glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity appears in the void volume, and the peak fraction was used for further studies. The LH-RH degrading endopeptidase is present in the fractions corresponding to an elution volume of 285–320 mL (K_{av} = 0.36). It was separated from residual aminopeptidase activities by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B and by adsorption chromatography on Hypatite C. It is recovered from the phenyl-Sepharose column in the range of 10–5 mS and from the Hypatite C column in the range of 25–40 mM phosphate. The final enzyme preparation was purified 900-fold (Table I) and free of other LH-RH degrading enzymes and amino- and carboxypeptidases. No substantial loss of enzyme activity could be detected after storage at −80 °C for 6 months in buffer D.

Determination of Physical Parameters. The initial velocity of the degradation of LH-RH by the endopeptidase is linearly proportional to the amount of protein and exhibits a pH optimum of 8.0 and a temperature optimum of 43 °C.

By cochromatographing the LH-RH degrading endopeptidase with standard enzymes of known molecular weight

Table I: Summary of Purification of the LH-RH Degrading Endopeptidase[a]

purifn step	total protein (mg)	total activity (units)	sp act. (units/mg of protein)	yield (%)	purifn (x-fold)
tissue extract (supernatant)	4590	109	0.02	100	1
$(NH_4)_2SO_4$ fractionation (35–65%)	1122	100	0.09	92	3
DEAE-cellulose chromatography	46	48	1.0	44	43
dialysis against deionized water	24.6	36	1.5	33	62
Ultrogel AcA 44 gel filtration	1.023	20	20	18	827
phenyl-Sepharose CL-4B chromatography	0.295	5	18	5	751
Hypatite C chromatography	0.095	2	22	2	925

[a] The enzyme preparations were preincubated with 1 mM 2-iodoacetamide and 0.5 mM diisopropyl fluorophosphate in buffer D containing 0.2 mM EDTA for 30 min at 0 °C prior to the determination of the [^{3}H]LH-RH degradation as described under Methods. One unit is defined as the enzymatic activity which degrades LH-RH at an initial velocity of 1 nmol/min under the incubation conditions used.

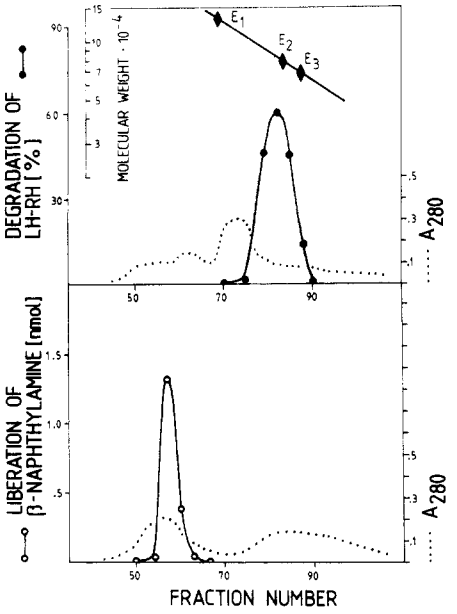

FIGURE 2: Gel filtration through Ultrogel AcA 44 columns. The LH-RH degrading endopeptidase (●) and the glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity (○) were further purified by gel filtration. For estimation of the molecular weight of the LH-RH degrading endopeptidase, it was cochromatographed with standard enzymes of known molecular weight (E_1, glucose-6-phosphate dehydrogenase; E_2, alkaline phosphate; E_3, malate dehydrogenase).

(glucose-6-phosphate dehydrogenase, M_r 130 000; alkaline phosphatase, M_r 80 000; malate dehydrogenase, M_r 70 000) on Ultrogel AcA 44 (Figure 2), we estimated a molecular weight of 83 000. The peak of the LH-RH degrading endopeptidase emerged slightly in front of that of the alkaline phosphatase.

The glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity is of high molecular weight. The liberation of β-naphthylamine proceeds with a maximal rate at pH 7.5 and is linearily proportional to time and the amount of protein.

Affinity of the LH-RH Degrading Endopeptidase toward LH-RH. The degradation of [^{3}H]LH-RH was measured in the presence of various amounts of unlabeled LH-RH. On the basis of a Lineweaver-Burk plot, the $K_I = K_M$ was calculated to be 180 μM at pH 7.4 and 37 °C.

Fragmentation of LH-RH and Synthetic Peptide Derivatives. Chymotrypsin is known to cleave LH-RH at the Trp^3-Ser^4 and Tyr^5-Gly^6 bonds (Matsuo et al., 1971). On ion-exchange chromatography on P 81 cellulose phosphate paper, LH-RH remains at the origin, whereas the N-terminal chymotryptic LH-RH fragments <Glu-His-Trp and <Glu-His-Trp-Ser-Tyr migrate with a R_f value of 0.5. The same fragmentation pattern could be observed after incubation of [^{3}H]LH-RH with the glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity. However, when [^{3}H]LH-RH was incubated with the LH-RH degrading endopeptidase, an additional fragment with $R_f = 0.62$ was formed.

For further identification of the cleavage products formed by the LH-RH degrading endopeptidase, the reaction mixture was resolved by high-voltage electrophoresis (Figure 3a), thin-layer chromatography, and ion-exchange chromatography (Figure 3b). <Glu-His-Trp-Ser-Tyr, Gly-Leu-Arg-Pro-Gly-NH_2, <Glu-His, and Trp-Ser-Tyr were identified by their amino acid composition (see the legend to Figure 3). The identity of Gly-Leu-Arg-Pro-Gly-NH_2 and <Glu-His was additionally confirmed by cochromatography with synthetic material. This fragmentation analysis indicates that the LH-RH degrading endopeptidase cleaves LH-RH at the His^2-Trp^3 and Tyr^5-Gly^6 bonds. <Glu-His-Trp could not be detected. Since this tripeptide is not metabolized by the endopeptidase (data not shown), it is concluded that the Trp^3-Ser^4 bond is not susceptible to hydrolysis by the LH-RH degrading endopeptidase.

Although the endopeptidase cleaves LH-RH at the carboxyl side of the aromatic amino acid tyrosine, liberation of β-naphthylamine from *N*-Cbz-Ser-Tyr-2-NNap and glutaryl-Gly-Gly-Phe-2-NNap could not be detected. *N*-Suc-Tyr-Gly-Leu-2-NNap and *N*-Suc-Phe-Gly-Leu-2-NNap, however, are degraded at comparable rates (data not shown). As shown in Figure 4, *N*-Suc-Phe-Gly-Leu-2-NNap is exclusively hydrolyzed at the Phe-Gly bond.

Kinetic Analysis. A kinetic analysis of LH-RH degradation and cleavage product formation by the endopeptidase is shown in Figure 5a. The formation of <Glu-His follows mixed kinetic functions. This effect is not due to enzyme activation during the incubation period, because the liberation of <Glu-His from <Glu-His-Trp-Ser-Tyr (see Figure 5b) exhibits first-order kinetics. With time, <Glu-His becomes the main product, whereas the concentration of <Glu-His-Trp-Ser-Tyr reaches a steady state and then declines. These results indicate that the LH-RH degrading endopeptidase preferentially cleaves the Tyr^5-Gly^6 bond of LH-RH and that <Glu-His is mainly liberated from <Glu-His-Trp-Ser-Tyr.

Effect of Functional Reagents. The enzymatic activities catalyzing the cleavage of the His-Trp and Tyr-Gly bonds could not be separated by the chromatographic methods used for enzyme purification. Furthermore, on polyacrylamide gel electrophoresis they comigrate with a relative mobility of 0.32 toward Bromphenol Blue. To substantiate the notion that these activities are attributable to one enzyme, the effect of functional reagents on the hydrolysis of LH-RH at the Tyr-Gly bond and of <Glu-His-Trp-Ser-Tyr at the His-Trp bond was examined (Table II). Both reactions are inhibited by SH-reactive agents (except 2-iodoacetamide) but remain unaf-

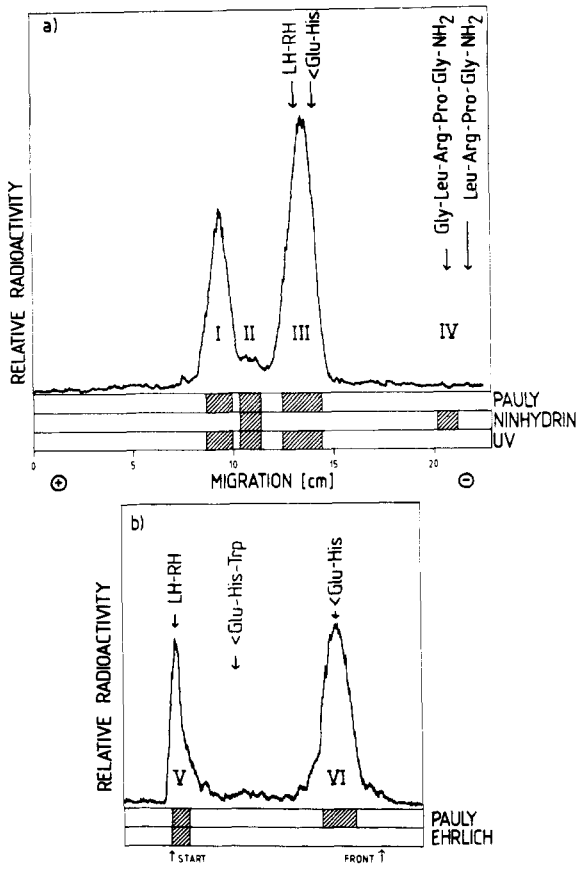

FIGURE 3: Fragmentation of LH-RH. The LH-RH degrading endopeptidase (0.5 mL) was incubated at 37 °C with 0.75 mg of LH-RH and 2.5 μCi of purified [^{3}H]LH-RH in the presence of 0.1 mM EDTA (0.55-mL final volume). After 2.5 h the reaction was stopped by the addition of 2 volumes of ice-cold methanol. The sample was kept at 0 °C for 30 min, and the precipitate was removed by centrifugation. The supernatant was concentrated in vacuo and applied to Whatman No. 3 paper, prewashed with 5% pyridine and 1% formic acid. The markers LH-RH, <Glu-His, Leu-Arg-Pro-Gly-NH_2, and Gly-Leu-Arg-Pro-Gly-NH_2 were spotted at the edge of the paper. High-voltage electrophoresis (60 V/cm), carried out at pH 1.9 (buffer E), was terminated, when migration of 6 cm was observed for the colored marker phenosafranine. The outer border of the sheet containing the marker substances and part of the resolved reaction mixture was sprayed with ninhydrin and Pauly's reagent. The strip containing the bulk of the resolved reaction mixture was scanned for radioactivity and viewed under UV light (a). The segments containing radiolabeled material or ninhydrin-positive material were eluted with 500 μL of 10% acetic acid each. The materials I and IV were subjected to acid hydrolysis and amino acid analysis. The ninhydrin-positive material II was further purified by thin-layer chromatography on silica gel plates [solvent system: 2-butanone–1-propanol–pyridine–H_2O–acetic acid (40:40:40:40:20 v/v/v/v/v)]. By spraying parts of the plate with ninhydrin and Ehrlich's reagent, we localized a component which had migrated with R_f = 0.83. It was eluted from the untreated part of the plate with 3 mL of 50% pyridine and subjected to acid hydrolysis and amino acid analysis. The material III was applied on CM-cellulose paper. At the edge of the paper LH-RH, <Glu-His, and <Glu-His-Trp were spotted. After development with water (ascending, 10 cm) and drying, the outer border of the sheet containing the marker substances and part of the resolved material was cut off and sprayed with Pauly's and Ehrlich's reagents. The strip containing the bulk of the resolved material was scanned for radioactivity (b). The radioactively labeled substances were eluted with 500 μL of 2 N ammonia solution and subjected to acid hydrolysis and amino acid analysis. Amino acid composition of the isolated material (the presence of Trp has been established by staining with Ehrlich's reagent or viewing under UV light): material I, Glu (0.82), His (0.98), Trp, Ser (1.1), and Tyr (1.1); material II, Trp, Ser (1.0), and Tyr (1.0); material IV, Gly (2.1), Leu (1.0), Arg (0.97), and Pro (0.93); material V, Glu (0.87), His (0.97), Trp, Ser (1.0), Tyr (1.0), Gly (2.1), Leu (1.0), Arg (0.96), and Pro (1.0); material VI, Glu (0.94) and His (1.06).

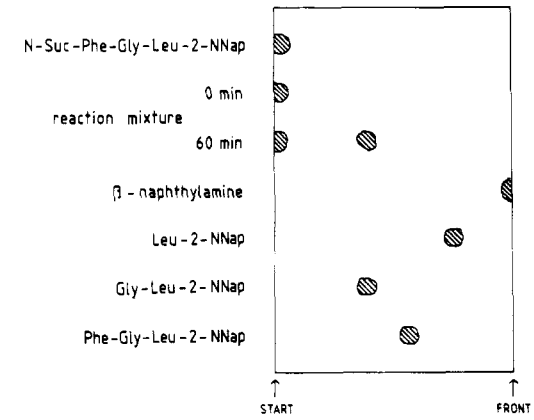

FIGURE 4: Fragmentation of *N*-Suc-Phe-Gly-Leu-2-NNap. *N*-Suc-Phe-Gly-Leu-2-NNap (20 nmol) was incubated at 37 °C with the LH-RH degrading endopeptidase in buffer C (final volume 100 μL). At given time intervals aliquots of the reaction mixture were withdrawn and spotted on silica gel F_{254} plates. β-Naphthylamine, Leu-2-NNap, Gly-Leu-2-NNap, and Phe-Gly-Leu-2-NNap were used as standards. After development (ascending, front 10 cm) in $CHCl_3$–CH_3OH–NH_3 aqueous (90:10:1 v/v/v), the plate was dried. β-Naphthylamine and β-naphthylamides were localized by viewing under UV light.

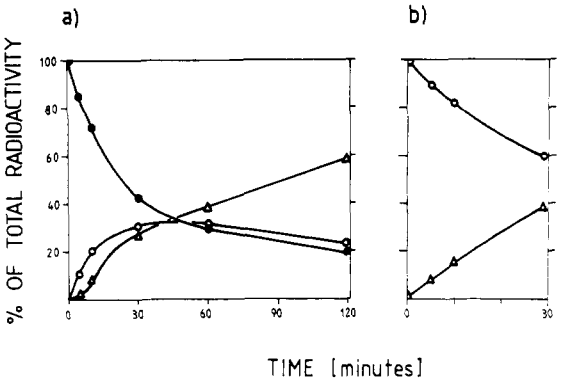

FIGURE 5: Kinetic analysis of [^{3}H]LH-RH and [^{3}H]<Glu-His-Trp-Ser-Tyr degradation. [^{3}H]LH-RH (a) and [^{3}H]<Glu-His-Trp-Ser-Tyr (b) were incubated with the LH-RH degrading endopeptidase. Aliquots of the reaction mixtures were resolved at given time intervals as described under Methods. The radioactivity in the segments containing LH-RH (●), <Glu-His-Trp-Ser-Tyr (○), and <Glu-His (△) was determined by scintillation counting.

fected by Ca^{2+}, *N*-tosylphenylalanine chloromethyl ketone, diisopropyl fluorophosphate, and EDTA. These properties differentiate the LH-RH degrading endopeptidase from the glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity and pancreatic α-chymotrypsin. The glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity is stimulated by Ca^{2+} and inhibited by diisopropyl fluorophosphate, but compared to α-chymotrypsin these effects are much less pronounced. *N*-Tosylphenylalanine chloromethyl ketone and *N*-ethylmaleimide are without any effect.

Discussion

Due to the presence of several LH-RH degrading enzymes as well as amino- and carboxypeptidases in tissue extracts, the initial cleavage products of LH-RH formed by one peptidase are subject to further degradation. Therefore, the separation of these enzymes from each other is a prerequisite for the determination of the initial cleavage sites. In this way hydrolysis of the <Glu^1–His^2 bond and the Pro^9–Gly-NH_2^{10} bond by a pyroglutamate aminopeptidase and a post-proline-cleaving enzyme has recently been shown. Previous reports on cleavage

Table II: Effect of Chemical Reagents[a]

test substance	concn (mM)	rel act. (%) LH-RH degrading endopeptidase: cleavage of <Glu-His-Trp-Ser-Tyr at the His-Trp bond	LH-RH degrading endopeptidase: cleavage of LH-RH at the Tyr-Gly bond	glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing act.	pancreatic α-chymotrypsin
no additive		100	100	100	100
Ca^{2+}	1.0	100	100	105	150
N-tosylphenylalanine chloromethyl ketone	0.03	100	100	100	48
diisopropyl fluorophosphate	0.3	100	100	75	0
p-(chloromercuri)benzoate	0.01	0	0	nd	nd
N-ethylmaleimide	0.1	25	20	100	100
2-iodoacetamide	1.0	100	100	nd	nd
EDTA	0.1	100	100	nd	nd

[a] Enzyme preparations without dithioerythritol were incubated at 0 °C for 15 min with the materials to be tested. The enzymatic reactions were started by addition of substrate. Cleavage of the His-Trp bond and Tyr-Gly bond was determined by using [^{3}H]<Glu-His-Trp-Ser-Tyr and [^{3}H]LH-RH as substrates. The glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity and pancreatic α-chymotrypsin were assayed with glutaryl-Gly-Gly-Phe-2-NNap as the substrate (for details, see Methods). nd, not determined.

of the Tyr^5–Gly^6 and/or the Gly^6–Leu^7 bond by neutral peptidases have not been ultimately confirmed, since either crude enzyme preparations had been used or the fragments had not unambiguously been identified. In the present study we have purified and characterized an endopeptidase which preferentially hydrolyzes the Tyr^5–Gly^6 bond of LH–RH.

The Tyr^5–Gly^6 and Trp^3–Ser^4 bonds of LH–RH are known to be susceptible to cleavage by pancreatic α-chymotrypsin. Although the LH–RH degrading endopeptidase described in this study also cleaves the Tyr–Gly bond, it differs from chymotrypsin in several important properties. The LH–RH degrading endopeptidase has a higher molecular weight (83 000) than α-chymotrypsin (25 000; Laskowski et al., 1966). In contrast to chymotrypsin, its activity is inhibited by SH-reactive agents but unaffected by Ca^{2+}, diisopropyl fluorophosphate, and *N*-tosylphenylalanine chloromethyl ketone. Significant differences are also obvious with regard to the cleavage specificity of the two enzymes. While α-chymotrypsin cleaves the Trp^3–Ser^4 bond of LH–RH, hydrolysis of this bond by the LH–RH degrading endopeptidase could not be observed. On the other hand, the endopeptidase cleaves the His^2–Trp^3 bond of LH–RH, which is not subject to hydrolysis by α-chymotrypsin. It is of interest to note that excretion of radioactive <Glu-His has been observed in studies on the fate of [^{3}H]LH–RH (Redding et al., 1974), but the involvement of the LH–RH degrading endopeptidase in the in vivo metabolism of LH–RH is yet unknown. The endopeptidase does not hydrolyze the fluorogenic chymotrypsin substrates glutaryl-Gly-Gly-Phe-2-NNap and *N*-Cbz-Ser-Tyr-2-NNap. This is probably due to the linkage of β-naphthylamine to the carboxyl group of the aromatic amino acid, since the degradation of *N*-Suc-Phe-Gly-Leu-2-NNap (via cleavage of the Phe–Gly bond) and of *N*-Suc-Tyr-Gly-Leu-2-NNap proceeds rapidly and at comparable rates.

Using the chymotrypsin substrate glutaryl-Gly-Gly-Phe-2-NNap, we have looked for chymotrypsin-like enzymes in pituitary extracts. An activity hydrolyzing this substrate could be detected and partially purified. Like pancreatic α-chymotrypsin, this activity is stimulated by Ca^{2+} and inhibited by diisopropyl fluorophosphate (albeit less), but it differs from chymotrypsin in its high molecular weight and insensitivity toward *N*-tosylphenylalanine chloromethyl ketone. This glutaryl-Gly-Gly-Phe-2-NNap hydrolyzing activity is capable of cleaving internal bonds of LH–RH, but it does not significantly contribute to the degradation of LH–RH by pituitary extracts.

Several enzymes cleaving peptides at the carboxyl side of aromatic amino acids have been described in the literature. The properties of the LH–RH degrading endopeptidase elucidated in this study clearly differentiate this enzyme from the calcium-activated proteinase (Guroff, 1964), neutral endopeptidase (Akopyan et al., 1979), cathepsin M (Marks, 1978), cation-sensitive neutral endopeptidase (Wilk & Orlowski, 1979), and chymotrypsin-like enzymes (Katunuma et al., 1975; Haas et al., 1978). It resembles the kininase A, which has been isolated from rabbit brain and which cleaves bradykinin at the Phe–Ser bond (Camargo et al., 1973), but further comparison has to await detailed investigations, especially with regard to the yet unknown substrate specifity of these two enzymes.

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Superoxide Dismutase from *Bacillus stearothermophilus*. Complete Amino Acid Sequence of a Manganese Enzyme†

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ABSTRACT: Superoxide dismutase from the thermophilic bacterium *Bacillus stearothermophilus* is dimeric with a molecular weight of 45 487. It contains one atom of manganese(III) per dimeric molecule [Brock, C. J., Harris, J. I., & Sato, S. (1976) *J. Mol. Biol. 107*, 175–178]. The subunits are identical, and the following primary structure comprising 203 amino acids has been determined:

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          10        20        30        40
PFELPALPYPYDALEPHIDKETMNIHHTKHHNTYVTNLNAALE-
      50        60        70        80
GHPDLQNKSLEELLSNLEALPESIRTAVRNNGGGHANHSLFWTI-
  90        100       110       120       130
LSPNGGGEPTGELADAINKKFGSFTAFKDEFSKAAAGRFGSGW-
          140       150       160       170
AWLVVNNGELEITSTPNQDSPIMEGKTPILGLDVWEHAYYLKY-
              180       190       200
QNRRPEYIAAFWNVVNWDEVAKRYSEAKAK
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The enzyme is identical in 60% of its residues with the *Escherichia coli* B manganese enzyme [Steinman, H. M. (1978) *J. Biol. Chem. 253*, 8708–8720]. Neither manganese enzyme has significant homology with the Cu/Zn superoxide dismutase from bovine erythrocytes. The secondary structures of the manganese enzymes predicted by McLachlan's method [McLachlan, A. D. (1977) *Int. J. Quantum Chem. 12*, 371–385] indicate that the eight-stranded β barrel of the Cu/Zn enzyme [Richardson, J. S., Thomas, K. A., Byron, H. R., & Richardson, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1349–1353] is absent from the manganese enzymes.

Superoxide dismutase catalyzes the dismutation of the $O_2^{-}\cdot$ radical to dioxygen and hydrogen peroxide. The enzyme is found in all organisms which metabolize oxygen. Its role may be to act as a defense against oxygen and superoxide toxicity (Fridovich, 1975).

The enzymes isolated from prokaryotes and mitochondria contain either manganese or iron. Both manganese and iron enzymes have been detected in *Escherichia coli* B (Dougherty et al., 1978). Only a manganese enzyme has been found in *Bacillus stearothermophilus*, but the existence of an iron enzyme cannot be excluded. The amino-terminal sequences of these enzymes are related to each other but not to the more widely studied enzymes found in the cytoplasms of eukaryotic cells (Harris & Steinman, 1977; Harris et al., 1980a; Walker et al., 1980a). These latter enzymes are dimeric and contain one Cu and one Zn per subunit (Fridovich, 1974). The dominant structural feature of the bovine erythrocyte Cu/Zn enzyme is an eight-stranded barrel of antiparallel β-pleated sheet (Richardson et al., 1975).

The manganoenzyme from the moderate thermophile *B. stearothermophilus* is dimeric and comprises two identical subunits of apparent molecular weight of ~20000 as estimated by gel electrophoresis in dodecyl sulfate and gel filtration in

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