Identification of Enzymatic Degradation Products of Luteinizing Hormone Releasing Hormone (LHRH)/[D-Ala^6] LHRH in Rabbit Mucosal Homogenates

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INTRODUCTION

Luteinizing Hormone Releasing Hormone (LHRH), a decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2, regulates the secretion of both luteinizing hormone and follicle stimulating hormone from the anterior pituitary (1). It is useful as a female contraceptive and treatment of infertility (2). According to recent reports (3), a single dose of LHRH and its analogs stimulates the release of pituitary gonadotrophins, multiple doses produce reversible pituitary desensitization, and this specific blockade of gonadotrophin provides the basis for the drug's efficacy in conditions dependent on sex hormone secretions. LHRH and its analogs are efficacious in the treatment of hormone sensitive prostate cancer and premenopausal breast cancer (4). In endometriosis, they produce a transient remission and in patients with uterine leiomyoma (4, 5), they reduce fibroid size.

LHRH and its analogs can be given effectively only by injection because, when taken orally, they are digested by the proteolytic enzymes in the GI tract and are metabolized by the liver. Consequently, the mucosal route of administration (nasal, buccal, rectal, conjunctival and vaginal) has recently been considered as an alternative to parenteral delivery for many peptide drugs because enzymatic degradation of these agents may be partly avoided. Another problem is that LHRH and its analogs have a relatively short duration of action and require repeated daily administration in order to achieve the desired effect. Under certain conditions it may also be possible to sustained the release of transmucosal administered drugs to prolong their duration of action. For clinical application, highly potent LHRH agonists have been developed as nasal sprays and vaginal pessaries for self administration. Unfortunately, the bioavailabilities or relative potencies of transmucosally administered peptides and proteins are usually quite low, unless absorption enhancers are coadministered. Metabolism and lack of membrane permeation can contribute to the poor bioavailability of transmucosally administered peptide and proteins. Metabolism might occur within the lumen of the rectal, nasal, buccal or vaginal cavities, at the surfaces of these membranes or within the cells.

Oyler (6) characterized the solution degradation product of Histrelin, an LHRH agonist and Marks & Stern (7) report the enzymatic mechanism for inactivation of LHRH in rat brain homogenates. Goren *et al.* (8) suggests that the degradation of LHRH in pituitary cytosol involves a two site specific peptidase, a Tyr^5-Gly^6 endopeptidase and Pro^9-Gly^{10}NH2 peptidase or postproline cleaving enzyme. Ueno (9) determined the LHRH analogue, leuprorelin and its metabolite in serum and urine. Orlowski *et al.* (10) reported that LHRH serves as a substrate for EP 24.15. This peptidase cleaves the central Tyr^5-Gly^6 bond in LHRH and the adjacent Gly^6-Leu^7 bond is cleaved by EP 24.11. In addition, they reported that angiotensin converting enzyme (ACE) produces LHRH$^{1-3}$ by cleavage of the Trp^3-Ser^4 bond of LHRH. Nevertheless, relatively little is known about the degradation profile of LHRH and the responsible enzymes in rectal, nasal and vaginal mucosa. The objective of this study was to investigate proteolysis of LHRH and [D-Ala^6] LHRH, a superactive analogue of LHRH in which Gly^6 is replaced by D-Alanine, in homogenates of the rectal, nasal and vaginal mucosa of the albino rabbit and to establish the optimal mucosal administration route of LHRH/[D-Ala^6] LHRH, based on presystemic metabolism.

MATERIALS AND METHODS

Material

LHRH, [D-Ala^6] LHRH, bovine serum albumin, tryptophan (Sigma Chemical Co.), disodium ethylene diamine tetra acetic acid (Tokyo Chemical Industries), sodium tauro 24,25-dihydrofusidate (California Biotechnology Inc. Mountain View, California), and a dye-binding assay kit (Bio-rad Laboratories, California) were used. All other materials were reagent or analytical grade.

Collection of Mucosa

Female albino rabbits weighing 2.0 to 2.5kg were anesthetized with sodium pentobarbital via a marginal ear vein. Mucosa were excised immediately in the following order: rectal, vaginal and nasal. Approximately 30min were required to excise all these mucosa from a single rabbit. The mucosa were rinsed in isotonic phosphate buffer, placed in boro silicate glass vials and stored at −32°C.

Rectal and vaginal mucosa were obtained by first exposing the respective luminal surface with a longitudinal incision followed by removing the mucosal tissue using a no.11 surgical blade. Nasal mucosa was obtained by making an incision along the length of the lateral wall of the nose on each dise of the nasal septum followed by cutting the nasal septum using surgical scissors and lifting the nasal bone fron-

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tally to expose the nasal cavity. Mucosal tissue was then carefully freed and removed from the underlying cartilage and bone.

Preparation of Rectal, Nasal and Vaginal Mucosal Homogenates of the Rabbit

Immediately before each experiment, mucosa were thawed to room temperature coolled with an ice bag and then homogenized in isotonic phosphate buffer (homogenizer : Ultra-Turex T 25, Janke & Kunkel GmbH, Germany). The homogenates were centrifuged at 3020 × g in a refrigerated (4°C) centrifuge (Himac CR 15D, Hitachi Co., Ltd., Japan) for 15min. The protein concentration of the supernatant was determined using a dye binding assay (11) with bovine serum albumin as the standard.

Enzymatic Degradation of LHRH/[D-Ala^6] LHRH in Mucosal Homogenates

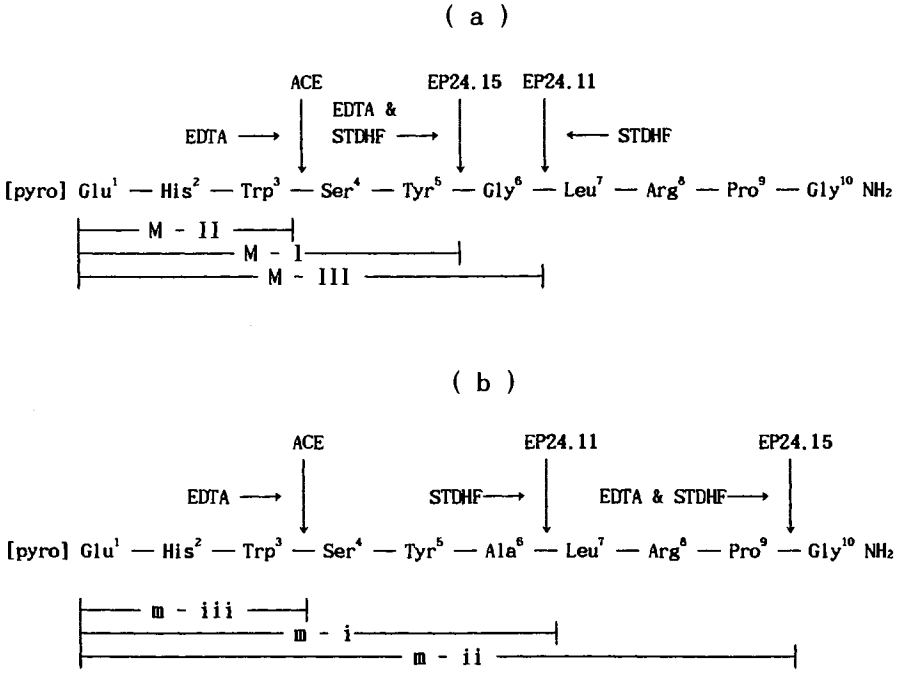
Tissue supernatant (100μl ; 1.25, 2.5 and 5mg protein/ml) was preincubated in 100μl of isotonic phosphate buffer (rectal and nasal: pH 7.4, vaginal: pH 6.0). After addition of an aliquot of LHRH/[D-Ala^6] LHRH (final concentration: 0.5mg/ml) solution, the mixture was incubated for 3hr at 37°C in a shaker water bath. To measure the effect of enzyme inhibitors on the degradation of LHRH/[D-Ala^6] LHRH, 2Na-EDTA(2%) or STDHF(1%) was added in solution before starting incubation. Subsequently the incubation mixture was sampled at predetermined time intervals. The reaction was stopped by addition of acetonitrile and vortexed for 10 seconds. After centrifugation for 15min. at 10,000 × g, the supernatant was evaporated under a stream of nitrogen. The residues were redissolved with 100μl mobile phase for analysis by HPLC.

Separation of Degradation Products by HPLC

Each experimental and control sample was subjected to reverse phase HPLC on a Lichrosorb RP-18 column (4 × 250 mm, 10 μm) interfaced with a Hitachi HPLC system consisting of an UV-VIS detector. The mobile phase was a mixture of acetonitrile and 0.6% ethanolamine in doubly deionized water (pH 3.0). The flow rate was 1ml/min. The column was first equilibrated with 14% acetonitrile for 5min, followed by a linear increase of acetonitrile to 30% for the next 8min and holding it at 30% final 7 min. Thereafter, the column was reequilibrated to 14% acetonitrile for 5min before the next injection. Degradation products in the eluate were monitored spectrophotometrically at 254nm.

Amino Acid Analysis of Degradation Products by PITC Method (12)

LHRH/[D-Ala^6] LHRH and their metabolites were collected from the HPLC solvent eluate and were lyophilized prior to acid hydrolysis. The samples were cleaned of impurities by Sep-Pak (C_{18}). Sample solutions were pipetted into sample tubes and dried. 20μl of the hydrolysis medium (6N HCl + 1% phenol) was pipetted into the reaction vial and sample tubes were then placed in the reaction vial. After 3 cycles of N_2 purging and evacuation, the reaction vial was placed in a hydrolysis oven and left at 110°C for 24 hours. Redrying of the samples were done by pipetting 10μl of a freshly prepared solution of ethanol-water-TEA (2:2:1, v/v) into each sample tube followed by evaporation to dryness. PITC derivatization was accomplished by pipetting 20μl of a freshly prepared solution of 10% PITC into the sample tubes. The composition of the 10% solution is ethanol-water-TEA-PITC (7:1:1:1 v/v). Tubes were left for 20 min. at room temperature. Subsequently, the samples were redried in a vac-



Scheme 1. Comparing the cleavage sites of LHRH (a) and [D-Ala^6] LHRH (b) by endopeptidases and the inhibition of 2Na-EDTA and STDHF.

uum to remove excess reagent. The amino acid components were analyzed using a Waters Associates Liquid Chromatograph equipped with a model 501 pump, 717 autosampler, 996 photodiode array detector, temperature control module, computer and printer. Chromatography was performed with a 3.9 mm ×15 cm reverse phase C_{18} column with a 10μm particle size. The dried samples were dissolved in 100μl of the starting HPLC buffer solution and 25μl of this solution was injected. The elution pattern was monitored at 254nm.

RESULTS

Identification of the Amino Acid Components of LHRH/[D-Ala^6] LHRH and their Metabolites

After incubation of LHRH/[D-Ala^6] LHRH in homogenates of rectal, nasal and vaginal mucosal homogenates, test samples were assayed by HPLC. Five to six degradation products of LHRH were detected (Figure 1(a)), whereas the number of degradation products of [D-Ala^6] LHRH were three to four (Figure 1(b)).

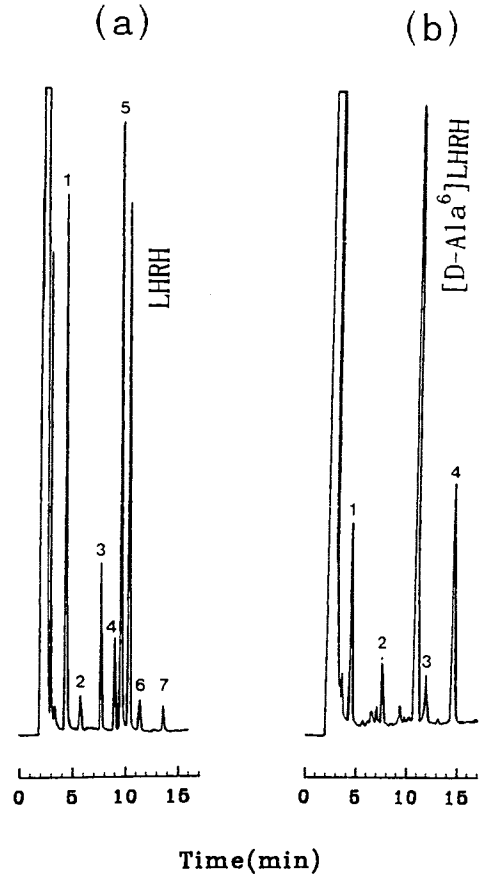


Fig. 1. HPLC separation of metabolites of LHRH (a) and [D-Ala^6] LHRH (b) in nasal mucosal homogenates. The incubation time of LHRH and [D-Ala^6] LHRH were 30 min and 90min, respectively. For details of the chromatographic conditions, see methods section. (a) The peaks of LHRH metabolites were identified as follows, (1), tryptophan; (2), M-VI (unknown); (3), M-II (LHRH$^{1-3}$); (4), M-III (LHRH$^{1-6}$); (5), M- I (LHRH$^{1-5}$); (6), M-V (unknown); (7), M-IV (unknown). (b) The peaks of [D-Ala^6] LHRH metabolites were identified as follows, (1), tryptophan; (2), m-iii ([D-Ala^6] LHRH$^{1-3}$); (3), m-ii ([D-Ala^6] LHRH$^{1-9}$); (4), m- i ([D-Ala^6] LHRH$^{1-7}$).

Amino acid analysis of the degradation products of LHRH indicated that M- I (retention time: 9.6min.) was LHRH$^{1-5}$, M-II (retention time: 7.7min.) was LHRH$^{1-3}$ and M-III (retention time: 9.0min) was LHRH$^{1-6}$, respectively. M-IV (retention time: 13.6min), M-V (retention time: 11.4 min) and M-VI (retention time: 5.7 min.) were formed in very low amounts so that their identity by amino acid analysis was not established. In the case of [D-Ala^6] LHRH, its degradation products were as follows; m- i (retention time: 14.6min) was [D-Ala^6] LHRH$^{1-7}$, m-ii (retention time: 12.0min.) was [D-Ala^6] LHRH$^{1-9}$, and m-iii (retention time: 7.6min) was [D-Ala^6] LHRH$^{1-3}$, respectively.

Enzymatic Degradation of LHRH in Rectal, Nasal and Vaginal Mucosal Homogenates

The degradation of LHRH after its incubation with rectal, nasal and vaginal mucosal homogenates was determined by measuring the disappearance of LHRH as well as the appearance of metabolites by HPLC. In rectal and nasal mucosal homogenate, M- I (LHRH$^{1-5}$) and M- II (LHRH$^{1-3}$) were the main degradation products of LHRH as well as a small amount of M-III(LHRH$^{1-6}$) (Fig. 2(a)), suggesting that the initial cleavage occurs at the Trp^3-Ser^4, Tyr^5-Gly^6 and/or Gly^6-Leu^7 bond, respectively. Although the profile of metabolites varied quantitatively and/or qualitatively at different time intervals in rectal and nasal mucosal homogenates, M-I, M-II and M-III appeared to be formed simultaneously. LHRH was rapidly degraded in rectal and nasal mucosal homogenates, and the half life was 17.9min and 44.4min, respectively. In vaginal mucosal homogenates, before the appearance of any other degradation products, M- I (LHRH$^{1-5}$) appeared at 15min after the incubation and was relatively stable compared to its appearance in rectal and nasal mucosal homogenates, where M- I (LHRH$^{1-5}$) was rapidly degraded to M- II (LHRH$^{1-3}$). M-II appears to undergo further degradation because the free Trp^3 is increased in incuabation mixture after 2hr incubation (not plotted). In addition, in vaginal mucosal homogenate M-IV (unknown metabolite) is appeared, while the M-III is not detected. Consequently, these results indicate that Tyr^5-Gly^6 is the major initial cleavage site on LHRH in several mucosal homogenates. The degradation profile of LHRH in rectal, nasal and vaginal mucosal homogenates based on protein concentration of the supernatants follows first order kinetics and accelerates with an increase in protein concentration. The degradation order was rectal > nasal > vaginal. Half lives of LHRH were calculated from the LHRH elimination curve and are presented in Table I. Half lives of LHRH in vaginal homogenates is 9–12 times longer than that in rectal, and 3–4 times greater than that in nasal. It would appear, the vaginal route is preferred for LHRH, based solely on presystemic metabolism.

Enzymatic Degradation Profile of [D-Ala^6] LHRH in Rectal, Nasal and Vaginal Mucosal Homogenates

The cleavage sites of [D-Ala^6] LHRH were different than that of LHRH. It was not cleaved at position six, but yielded to m- i ([D-Ala^6] LHRH$^{1-7}$), m-ii ([D-Ala^6] LHRH$^{1-9}$) as major metabolites with a small amount of m- iii ([D-Ala^6] LHRH$^{1-3}$), indicating that initial cleavage occurs at the Leu^7-Arg^8, Pro^9-Gly^{10} and Trp^3-Ser^4 bond, respectively. Fig. 2(b)

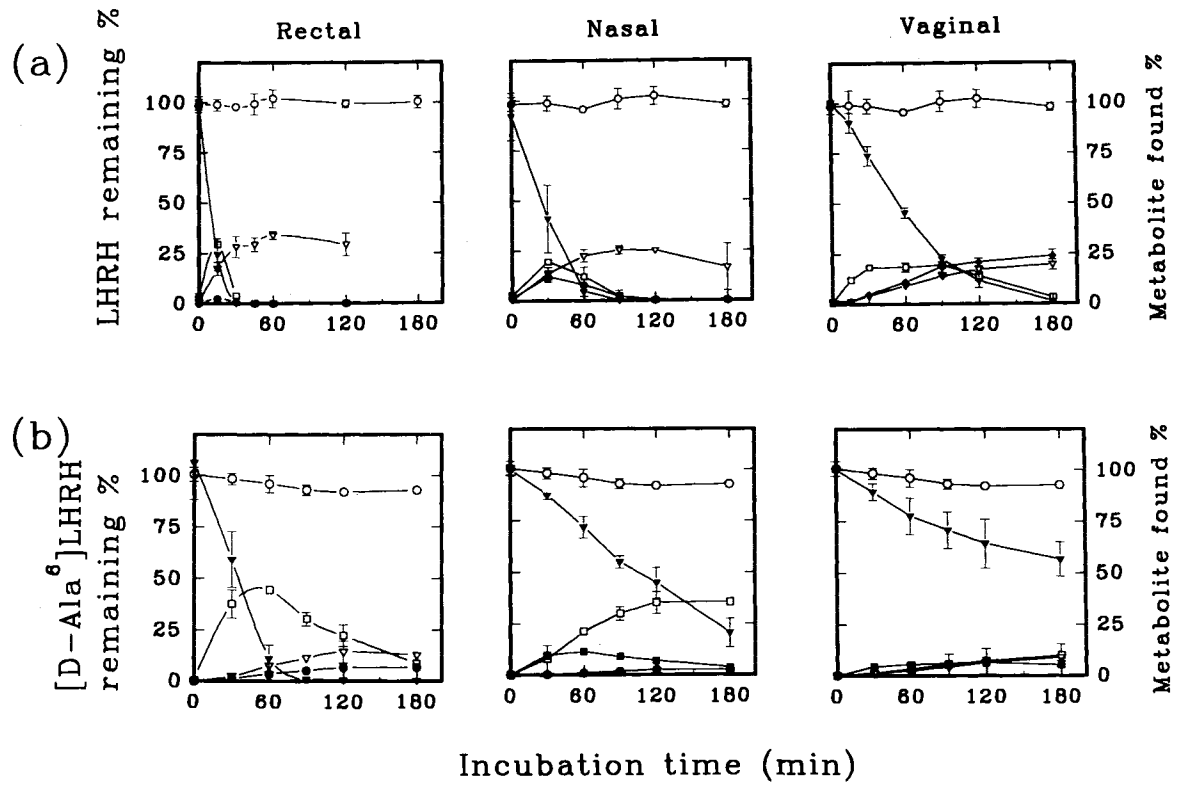


Fig. 2. Proteolysis profile of LHRH/[D-Ala^6] LHRH and the formation of metabolites in various mucosal homogenates. Each point represents the mean ± S.E. of three experiments. The tissue protein concentration was 2.5mg/ml. Key: (a) (○), LHRH control (in the absence of tissue protein); (▼), LHRH; (□), M- I (LHRH$^{1-5}$); (▽), M-II (LHRH$^{1-3}$); (●), M-III (LHRH$^{1-6}$); (◆), M-IV (unknown). (b) (○), [D-Ala^6] LHRH control (in the absence of tissue protein); (▼), [D-Ala^6] LHRH; (□), m- i ([D-Ala^6] LHRH$^{1-7}$); (■), m- ii ([D-Ala^6] LHRH$^{1-9}$); (▽), m-iii ([D-Ala^6] LHRH$^{1-3}$); (●), m-iv (unknown)

shows the degradation profile of [D-Ala^6] LHRH in rectal, nasal, and vaginal mucosal homogenates. In rectal mucosal homogenates, [D-Ala^6] LHRH was rapidly degraded to m- i ([D-Ala^6] LHRH$^{1-7}$) to a large extent, whereas m- ii ([D-Ala^6] LHRH$^{1-9}$) was not detected. In addition, small amounts of m-iii ([D-Ala^6] LHRH$^{1-3}$) and, m-iv (unknown) were detected. In nasal mucosal homogenates, the degradation of [D-Ala^6] LHRH was slower than that in rectal mucosal homogenates, but m- ii ([D-Ala^6] LHRH$^{1-9}$) was detected. The degradation product m- ii ([D-Ala^6] LHRH$^{1-9}$) might not be detectable prior to degradation to m- i ([D-Ala^6] LHRH$^{1-7}$). That is to say, the degradation enzyme (i.e EP 24.11) of [D-Ala^6] LHRH in rectal mucosa is more abundant than in nasal mucosa, and degradation from m- ii to m- i may be rapid since m- ii was not detected in the incubation mixture. On the other hand, degradation of [D-Ala^6] LHRH in vaginal mucosal homogenates was slower than that in rectal or nasal mucosal homogenates. A large amount of intact [D-Ala^6] LHRH was detected compared with rectal or nasal mucosal homogenates. The degradation products, m- i, m- ii and iii, were detected to a lesser extent. The degradation of [D-Ala^6] LHRH in rectal, nasal and vaginal mucosal homogenates according to protein concentration of the incubation mixture was similar to, but slower, than that of LHRH. Half lives of [D-Ala^6] LHRH calculated from the [D-Ala^6] LHRH elimination curve are presented in Table I.

Table I. Half Life (min) of LHRH and [D-Ala^6] LHRH After Incubation in Various Mucosal Homogenates[a]

Drug	Protein conc. (mg/ml)	Rectal	Nasal	Vaginal
LHRH	1.25	7.5 ± 0.8	19.7 ± 0.6	90.9 ± 10.2
	2.5	2.7 ± 0.8	12.0 ± 1.1	29.9 ± 8.5
	5.0	1.7 ± 0.5	5.5 ± 1.8	15.0 ± 9.2
[D-Ala^6] LHRH	1.25	22.7 ± 3.6	132.7 ± 5.2	261.7 ± 6.0
	2.5	17.9 ± 0.3	87.7 ± 8.7	216.5 ± 15.3
	5.0	12.7 ± 0.4	21.7 ± 3.4	30.9 ± 10.9

[a] Each value represents the mean ± S.E. of three different experiments.

Effects of 2Na-EDTA or STDHF on the Degradation of LHRH/[D-Ala^6] LHRH

Addition of 2Na-EDTA to the incubation mixture significantly inhibited LHRH degradation and completely abolished the formation of both M- I (LHRH$^{1-5}$) and M-II (LHRH$^{1-3}$) whereas it did not inhibit the formation of M-III-(LHRH$^{1-6}$). In the presence of STDHF, the formation of M-I (LHRH$^{1-5}$) and M-III(LHRH$^{1-6}$) was completely abolished and M- II(LHRH$^{1-3}$) was increased (Figure 3(a)). The half-life of LHRH increased from 2.7min to 169.5min (2Na-EDTA) and 699.8min (STDHF) in rectal mucosl homogenates (protein conc. 2.5mg/ml).

The degradation of [D-Ala^6] LHRH was inhibited by addition of 2Na-EDTA. 2Na-EDTA inhibited the formation of m- ii and m-iii without inhibiting that of m- i. On the other hand, the degradation of [D-Ala^6] LHRH was significantly inhibited by addition of STDHF and the metabolites were at the threshold of detection (Fig. 3(b)). Incubation of [D-Ala^6] LHRH and either 2Na-EDTA or STDHF led to an increase in half-life of [D-Ala^6] LHRH from 17.9min to 362.5min and 614.1min in rectal mucosl homogenates (protein conc. 2.5mg/ml), respectively.

DISCUSSION

Orlowski *et al.* (10) isolated endopeptidase 24.11 (EP 24.11) from the pituitary and brain. They also isolated endopeptidase 24.15 (EP 24.15) from the soluble fraction of brain homogenates. And reported that LHRH serves as a substrate for EP 24.15. This peptidase cleaves the central Tyr^5-Gly^6 bond in LHRH and the adjacent Gly^6-Leu^7 bond is cleaved by EP 24.11. In addition, they reported that angiotensin converting enzyme (ACE) produces LHRH$^{1-3}$ by cleavage of the Trp^3-Ser^4 bond of LHRH. Amino acid analysis of LHRH in the present study revealed that LHRH may be degraded by EP 24.15, EP 24.11 and ACE in rectal, nasal and vaginal mucosal homogenates as well as pituitary and brain. The profile of metabolites formation indicate that EP 24.15 exists in each mucosal homogenate with the order of rectal > nasal > vaginal and EP 24.15 is the primary LHRH degrading enzyme. In the present study, as previously described, the formation of LHRH$^{1-5}$ and LHRH$^{1-3}$ are inhibited by the addition of 2Na-EDTA to the incubation medium. In the presence of STDHF, the formation of LHRH$^{1-5}$ and LHRH$^{1-6}$ were completely abolished (Fig. 3(a)). These results indicate that 2Na-EDTA acts as an inhibitor of EP 24.15 & ACE whereas STDHF acts as an inhibitor of EP 24.15 and EP 24.11. The cleavage sites of LHRH by several endopeptidase and the inhibition effects of 2Na-EDTA and STDHF were clarified in scheme 1(a).

Flouret *et al.* (13) reported that superactive analogs of LHRH in which Gly^6 was replaced by a D-amino acid are resistant to degradation by both EP 24.11 and EP 24.15. Lasdun (14) reported that two superactive analogs of LHRH, [D-Trp^6] LHRH and [D-Leu^6, Des-Gly-NH$_2$(10)] LHRH ethylamide, are resistant to degradation by EP 24.15, due to the presence of a D-amino acid in position six. Nakamura (15) also reported that relative in-vivo biological ac-

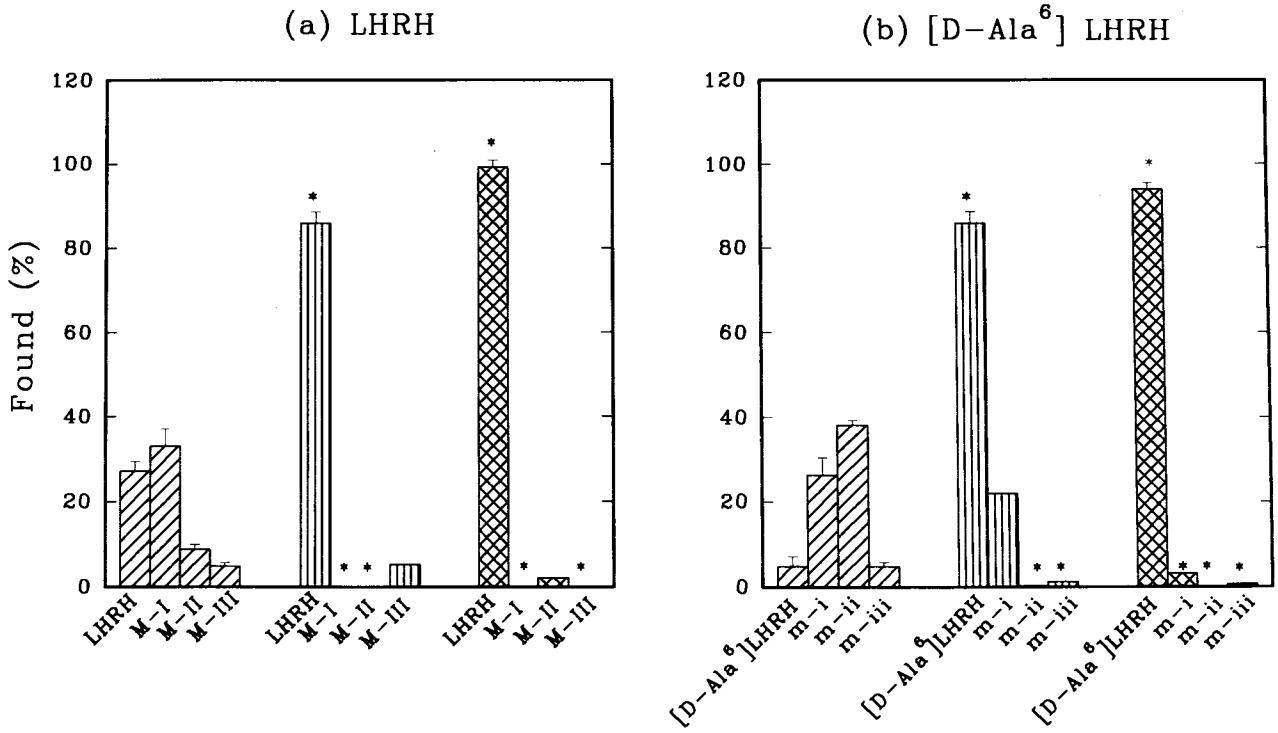


Fig. 3. Inhibitory effects of 2Na-EDTA (2%) and STDHF (1%) on the LHRH (a) or [D-Ala^6] LHRH (b) proteolysis in rectal mucosal homogenates. The incubation time of LHRH and [D-Ala^6] LHRH were 30 min and 120 min, respectively. Each point represents the mean ± S.E. of three experiments. The tissue protein concentration was 1.0mg/ml. M- I, M-II, M-III, m-i, m-ii, and m-iii represent LHRH$^{1-5}$, LHRH$^{1-3}$, LHRH$^{1-6}$, [D-Ala^6] LHRH$^{1-7}$, [D-Ala^6] LHRH$^{1-9}$, and [D-Ala^6] LHRH$^{1-3}$, respectively. * Significantly different from the control ($p < 0.01$). Key: (▨), control; (▩), 2Na-EDTA (2%); (▩), STDHF (1%).

tivity of [D-Ala^6] LHRH is 4.8 times higher than mammalian LHRH (mGnRH). The results of our study also demonstrated that the half lives of [D-Ala^6] LHRH is 3–7 times greater than LHRH (Table I). After addition of 2Na-EDTA to the incubation medium, only [D-Ala^6] LHRH$^{1-7}$ was produced and STDHF almost completely inhibited the formation of metabolites of [D-Ala^6] LHRH (Fig. 3(b)). These findings indicate that 2Na-EDTA inhibited EP 24.15 & ACE without inhibition of EP 24.11, resulting in the formation of m- i ([D-Ala^6] LHRH$^{1-7}$). Since 2Na-EDTA inhibited the formation of [D-Ala^6] LHRH$^{1-9}$, and STDHF inhibited the formation of [D-Ala^6] LHRH$^{1-7}$ & [D-Ala^6] LHRH$^{1-9}$, [D-Ala^6] LHRH$^{1-9}$ is considered to be produced by EP 24.15. Scheme 1(b) illustrate the identified sites of cleavage and the inhibition effects of 2Na-EDTA and STDHF. We noticed that ACE produced equally Glu-His-Trp fragment in LHRH and [D-Ala^6] LHRH. It is clear that [D-Ala^6] LHRH is resistant to degradation by EP 24.15 based on the extent of metabolite formation and the degradation rate of [D-Ala^6] LHRH. Although only a small amount of [D-Ala^6] LHRH$^{1-9}$ was detected in the incubation medium, we are not certain that EP 24.11, a secondary degrading enzyme of LHRH, is the primary degrading enzyme of [D-Ala^6] LHRH because we have no certainty whether EP 24.11 cleaves the Leu^7-Arg^8 bond of [D-Ala^6] LHRH$^{1-9}$ and/or the intact [D-Ala^6] LHRH. In any case, the smaller amount of [D-Ala^6] LHRH$^{1-9}$, as compared to LHRH$^{1-5}$, may be due to resistance of [D-Ala^6] LHRH to EP 24.15.

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