END GROUP AND CARBOHYDRATE ANALYSES OF EQUINE LH

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SUMMARY. The terminal amino acid residues and carbohydrate composition of a highly purified equine luteinizing hormone (LH) preparation were determined. The N-terminal amino acids were serine and phenylalanine while the C-termini were determined to be serine and isoleucine. The carbohydrate analyses showed high amounts of sialic acid.

There have been few reports on the extraction and purification of equine gonadotropins (1-3). None of these preparations have been extensively characterized due to contamination. Braselton and McShan (4) reported a procedure for obtaining highly purified equine luteinizing hormone (LH). The present study reports the characterization of the equine LH molecule with respect to end groups and carbohydrate content.

EXPERIMENTAL

Preparation of the LH. Equine LH was prepared according to a modification of the method of Braselton and McShan (4). A 0.05 M, pH 6.75 ammonium acetate buffer was used in the final gel filtration step instead of the 0.05 M, pH 7.0 phosphate buffer in 0.1 M NaCl. The sample was desalted by ultrafiltration and then freeze-dried. Following lyophilization, the equine LH was dried to constant weight in a vacuum desiccator over anhydrous calcium sulfate at room temperature.

The equine LH preparations were assayed by the ovarian ascorbic acid depletion method of Parlow (5). They were found to contain an average of 2.6 units of NIH-LH-S1 per mg.

Chemicals. Orcinol was purified before use. The other chemicals were reagent grade.

N-terminal analysis. N-terminal determinations were made using two methods of analysis. The dansyl chloride procedure of Gros and Labouesse (6) was used to determine the N-terminal residues of desialyzed equine LH. Desialylation was achieved by incubation in 0.1 N H_2SO_4 at 80° for one hour. Crystalline bovine insulin (Eli Lilly, Indianapolis, Indiana) was used throughout the determinations as the standard protein in order to evaluate the reliability of the method. Correction factors were determined from standard dansyl amino acids and applied to recovery values of LH.

Leucine aminopeptidase (LAP) was also used in the analysis of the N-terminal amino acids. A time-course study was conducted using an enzyme to substrate ratio of 1:30 (mg:mg). The hormone was dissolved in 0.01 M Tris buffer, pH 8.5, containing 0.005 M $MgSO_4$ and then denatured by heating. After activating the enzyme in the same buffer for 30 to 45 minutes, LAP was added to the hormone and digestion was performed at 40°. Aliquots were removed at selected time intervals, heated to stop enzyme action and frozen until analysis.

C-terminal analysis. Hydrazinolysis was performed according to a modification of the procedure of Reichert et al. (7). Samples of 100 nmoles of hormone were dried in a vacuum oven at 80° over silica gel for 20 hours. Anhydrous hydrazine (0.5 ml) was added, the tube evacuated and sealed. Hydrazinolysis was allowed to continue for 20 hours at 80°. The hydrazinolyzate was dried in a desiccator over concentrated H_2SO_4 and KOH. The sample was dissolved in 3 ml of water and extracted with 1 ml of benzaldehyde for time periods of 0.5, 1.0 and 3.0 hours. The mixtures were shaken constantly on a Vortex Mixer and centrifuged. This step was followed by extraction with 2 ml of ethyl acetate, followed

by 3 ml of ether. The remaining ether was removed by heating the water phase at 60° for 15 minutes. The sample was then frozen until analysis. A Beckman amino acid calibration mixture was subjected to the conditions of hydrazinolysis in order to obtain correction factors for destruction.

Carboxypeptidase A digestion was performed as another method to determine the C-termini. The hormone was dissolved in 0.04 M borate buffer, pH 8.15, and then heated for denaturation. The enzyme was added to give a final enzyme to substrate ratio of 1:40 (nmole:nmole). Digestion was allowed to continue at 37°. At predetermined time intervals, aliquots were removed. The reaction was stopped by adding five volumes of 0.2 M sodium citrate, pH 2.2, to the aliquot. The samples were then frozen until analysis.

Carbohydrate Analysis. Total hexose was determined by the orcinol-H_2SO_4 procedure of Winzler (8) as modified by Francois et al. (9). The hexosamines were quantitated on a Beckman Model 120C Amino Acid Analyzer using a 50 minute buffer change. The LH was hydrolyzed under nitrogen in 4 N HCl (1 mg/ml) at 100° for 4 hours to release the hexosamines. The acid was removed in vacuo over KOH and H_2SO_4 at 0°. The method of Dische and Shettles (10) as utilized by Dische (11) was used to determine fucose. Sialic acid was determined by the thiobarbituric acid method of Warren (12) following hydrolysis in 0.1 N H_2SO_4 at 80° for one hour.

RESULTS

N-terminal Amino Acids. The results from N-terminal analysis with dansyl chloride indicated serine and phenylalanine as the N-termini (Table I). These were found in corrected molar ratios of 0.64 and 0.68 respectively.

The results from LAP digestion (Table II) support those found by the dansyl chloride method. Serine and phenylalanine were released from the equine LH molecule, approaching molar quantities of

Table I. N-terminal Amino Acids of Desialylated Equine LH as Determined by Dansyl Chloride.

Amino Acid	Recovery*
Serine	0.64
Phenylalanine	0.68

*Values are expressed in corrected molar ratios based on MW of 44,500.

Table II. N-terminal Amino Acids Released from Equine LH by Leucine Aminopeptidase Digestion.*

Amino Acid	Digestion Time (minutes)					
	30	90	180	300	540	720
Serine	0.50**	0.54	0.58	0.60	0.61	0.68
Phenylalanine	0.12	0.21	0.30	0.39	0.50	0.65
Glutamic Acid	0.10	0.14	0.15	0.16	0.19	0.25
Glycine	0.32	0.32	0.31	0.35	0.37	0.39
Aspartic Acid	0.22	0.24	0.23	0.20	0.24	0.22
Alanine	0.23	0.28	0.21	0.27	0.29	0.24

* Enzyme to substrate ratio of 1:30 (mg:mg).
** Values are expressed in molar ratios based on MW of 44,500.

0.70 and 0.65 respectively. Other amino acids were recovered but only glutamic acid was released kinetically. The presence of the others may be explained by the heterogeneity of equine LH reported by Braselton and McShan (4). It is also possible that these are bound amino acids (13) and/or contaminants.

C-terminal Amino Acids. Results from the hydrazinolysis study indicated serine and isoleucine as the C-terminal amino acids, as shown in Table III. These results were supported by those found with carboxypeptidase A digestion. Serine and isoleucine were released kinetically by the enzyme and approached equimolar quantities. The data from this experiment are shown in Table IV.

Table III. C-terminal Amino Acids of Equine LH as Determined by Hydrazinolysis.*

Amino Acid	Recovery**
Serine	1.15
Isoleucine	0.63
Glycine	0.30

*Hydrazinolysis for 20 hours at 80°.
**Values are expressed in molar ratios based on MW of 44,500 and are corrected for destruction.

Table IV. C-terminal Amino Acids Released from Equine LH by Carboxypeptidase A Digestion.*

	Digestion Time (minutes)						
Amino Acid	5	15	30	60	180	240	480
Isoleucine	----	0.20**	0.26	0.55	0.63	0.83	0.95
Serine	0.20	0.21	0.26	0.30	0.50	0.62	0.80
Glycine	0.13	0.15	0.17	0.30	0.47	0.40	0.45

* Enzyme to substrate ratio of 1:40 (nmole:nmole).
** Values are expressed in molar ratios based on MW of 44,500.

Carbohydrate content. The results from the carbohydrate analyses are given in Table V. These are compared with the carbohydrate contents of LH molecules of other species. The most significant finding of these determinations is the high sialic acid content.

DISCUSSION

This report represents a partial characterization of the equine LH molecule. End group analyses have indicated the presence of two amino- and two carboxy- terminal amino acids. This fact suggests that the equine LH molecule consists of subunits. Additionally, the results, when compared to those found with other horse gonadotropins, suggest a structural similarity. Papkoff and Schams (14)

Table V. Carbohydrate Content of Equine LH: Comparison with Other Species.*

Carbohydrate	Equine	Human (21)	Ovine-Fr3 (22)	Bovine (23)
Hexose	8.30	12.1	6.55	5.3
Fucose	0.57	--	1.19	0.9
Total Hexosamine	8.61**	5.0	8.44**	7.0
Glucosamine	4.53	--	5.39	--
Galactosamine	3.10	--	2.38	--
Sialic Acid	7.92	1.6	0.63***	0.0

* Values are expressed in gm/100gm of glycoprotein.

** Total hexosamine determined by the method of Rondle and Morgan (24) whereas individual values of glucosamine and galactosamine were determined by amino acid analysis.

*** Unpublished data from this laboratory.

reported phenylalanine as the N-terminal of both pregnant mare serum gonadotropin (PMSG) and pregnant mare endometrial cup gonadotropin (PMEG) while Nuti (unpublished data) reported similar results with end group analysis of equine FSH. Further evidence for this similarity was demonstrated by the difficulty experienced in separating equine gonadotropins (15) and also by the inactivation of both equine LH and FSH by chymotrypsin digestion (16). The extent of the similarity among equine gonadotropins awaits further analysis.

The most significant finding of the carbohydrate analyses was the high sialic acid content. It is conceivable that this high sialic acid content may be responsible for the long biological half-life of equine LH (17), especially in view of the recent studies concerning the relation of sialic acid content and biological survival of glycoprotein hormones (18-20). Further investigations should give more insight into this possibility.

This report represents the first step in the elucidation of the structure of equine LH. Future studies may be facilitated by

subunit dissociation and subsequent analyses.

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