Low-molecular-weight Inhibin from Sheep, Human, Rat, and Chicken Testes

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Low-molecular-weight peptides with inhibin activity have been isolated from sheep, human, rat, and chicken testes by simple gel filtration. These peptides, isolated from avian and mammalian species, suppressed the postcastration rise of serum FSH levels in adult male rats by 34 to 45% and appeared to be biologically and chromatographically similar.

Key words: inhibin, testes, avian, mammalian.

Inhibin from the testes and testicular secretions of several species has been isolated and characterized (Lee et al, 1974, 1977; Lugaro et al, 1973, 1974; Franchimont et al, 1975a, b, 1977, 1978; Keogh et al, 1976; Moodbidri et al, 1976; Nandini et al, 1976; Baker et al, 1976; Setchell et al, 1977; Chari et al, 1978a, b; Thakur et al, 1978; Sheth et al, 1979; Shashidharamurthy et al 1979; Cahoreau et al, 1979; Vijayalakshmi et al 1980a,b). Although it is generally agreed that inhibin is a water-soluble protein, there is considerable controversy as to its molecular weight, with the reported values ranging from 1500 to 160,000 daltons for different preparations. The authors have previously reported the isolation of low-molecular-weight (1500 daltons) inhibin from ovine testes and ovaries (Sheth et al, 1979; Vijayalakshmi et al, 1980a). The present study examines testicular tissues from other species, ie, man, rat, and chicken, for the presence of a similar peptide.

Materials and Methods

Human testes were obtained at autopsy from a local hospital. Ram and chicken testes were procured from local meat and poultry suppliers, while rat testes were obtained from adult Holtzman rats. The testes were detunicated and freed from the epididymides. Using a Waring-type blender, 40% homogenates were prepared in 0.01 M phosphate buffer, *p*H 7.4. The homogenates were subjected to ultracentrifugation at 100,000 × g for 1 hour to obtain cytosol.

Chromatography of Cytosol

Fifty ml of cytosol containing approximately 1 g of protein was subjected to gel filtration on a Sephadex G-100 column (5 × 90 cm), using 0.01 M phosphate buffer, *p*H 7.4, for elution. The column was eluted at the rate of 40 ml/hr, and 10-ml fractions were collected. Fractions exhibiting inhibin activity were concentrated by ultrafiltration in an Amicon filtration cell fitted with a UM-2 membrane. The retentate was subjected to chromatography on a Sephadex G-25 column (2.5 × 38 cm) equilibrated and eluted with 0.01 M phosphate buffer, *p*H 7.4. The column was eluted at the rate of 20 ml/hr, and the eluate was collected in 3-ml fractions.

The protein content of the chromatographic fractions was determined by the method of Lowry et al (1951).

Assay of Inhibin Activity

Inhibin activity of the different chromatographic fractions was assessed by their ability to suppress circulating levels of FSH in castrated adult male rats.

Ninety-day-old Holtzman rats, bilaterally castrated two weeks prior to assay, were injected intramuscularly once daily for three days with saline or the test material. Four hours after the last injection, the animals were bled, and the serum was collected. The serum levels of FSH and LH were determined by radioimmunoassay using NIAMDD systems, and the results were expressed in terms of NIAMDD-Rat-FSH-RP-1 and NIAMDD-Rat-LH-RP-1, respectively.

Serum FSH and LH levels in groups of animals treated with the test materials were compared with those in the saline-treated group. Significant suppression of FSH

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Submitted for publication October 10, 1980; revised version received February 11, 1981; accepted for publication May 29, 1981.

0196-3635/82/0300/0140/$00.70

levels was taken as an index of inhibin activity. All Statistical evaluations were performed using Student's *t* test. The index of precision (λ) for the assay was 0.169. The relative potencies of different inhibin preparations were calculated by the method of Borth (1960).

Results

In all species studied, fractionation of testicular cytosol on Sephadex G-100 columns resulted in their resolution into three fractions (Fig. 1). The patterns of elution were nearly identical, and inhibin activity was found to reside in the most retarded fraction (Rm-3, Hm-3, Rt-3, and Ch-3), irrespective of species. The fractions eluting in the void volume (Rm-1, Hm-1, Rt-1, and Ch-1) suppressed LH to the extent of 24 to 37% without any apparent effect on FSH. This observation, although interesting, is only peripherally related to the authors' main interest, the isolation of inhibin, and therefore will not be discussed in detail.

The shoulder between the two main peaks, identified as fraction 2 (Rm-2, Hm-2, Rt-2, and Ch-2), did not affect either FSH or LH levels (Table 1).

The rechromatography of fractions exhibiting inhibin activity (Rm-3, Hm-3, Rt-3, and Ch-3) on Sephadex G-25 columns again generated nearly identical patterns of elution consisting of five peaks (Fig. 2). Inhibin activity was identified in the fractions (Rm-3II, Hm-3II, Rt-3II, and Ch-3II) immediately following the void volume in all cases (Table 2). These fractions were referred to as ram, human, rat, and chicken inhibin, respectively, and were obtained in yields of about 250 mg per kg of testicular tissue. Fraction Rm-3II is equivalent to TFR II, the authors' previously reported ram testicular inhibin, which has been characterized as a heat-stable, trypsin-sensitive peptide with a molecular weight of about 1500 daltons (Vijayalakshmi et al 1980a).

In the absence of a standard or reference preparation, human, rat, and chicken testicular inhibin preparations were compared with the ram testicular inhibin preparation which has been characterized to some extent (Moodbidri et al, 1976; Sheth et al 1979; Vijayalakshimi et al 1980a, b; Moodbidri et al, 1981.). It was observed that the human preparation was 1.75 times as active as the ram preparation, while those from rats and chickens were 1.44 and 0.65 times as active respectively (Table 2). The differences in the slopes of the dose response curves for different preparations were negligible.

Discussion

The authors' previous study (Moodbidri et al, 1976; Sheth et al, 1979; Vijayalakshmi et al, 1980a) demonstrated the existence in ovine testes and ovaries of a small peptide (1500 daltons) with inhibin activity. Here, the authors demonstrate the presence of a similar low-molecular-weight peptide with inhibin activity in man, rat, and chicken. The comparative data of the biological and chromatographic behavior of inhibin preparations from those species indicate similarity.

Several molecular species of inhibin with molecular weights ranging from 5000 to 160,000 daltons have been identified from ram rete testis fluid (Baker et al, 1976; Franchimont et al, 1977, 1978; Davies et al, 1978; Cahoreau et al, 1979). Fran-

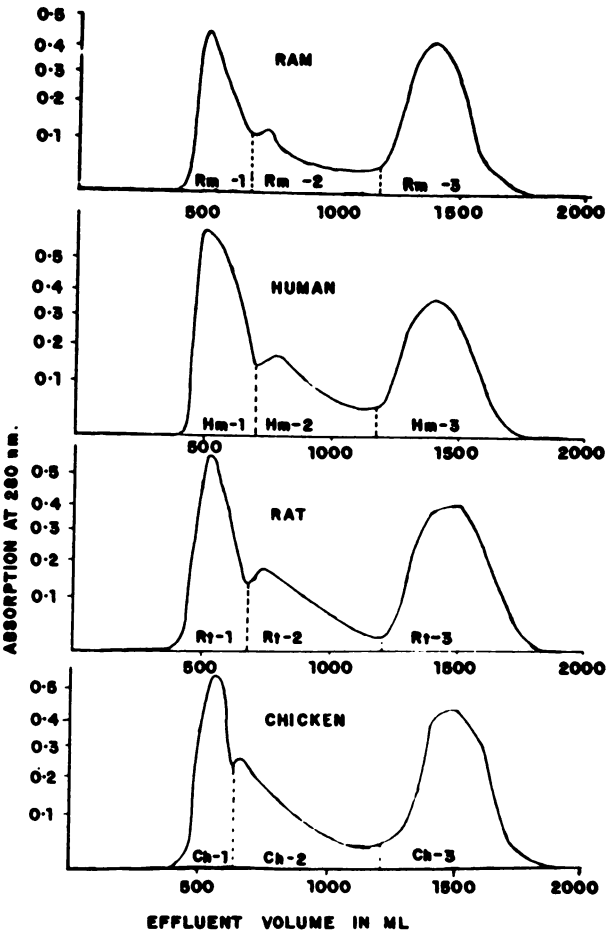

Fig. 1. Chromatographic elution profile of ram, human, rat, and chicken testicular cytosol on Sephadex G-100 column (5 × 90 cm). The elution buffer was 0.01 M phosphate buffer, *p*H 7.4. The flow rate was 40 ml/hr, and the fraction volume was 10 ml.

TABLE 1. Effect of Cytosol Fractions on Serum FSH and LH Levels in Castrated Male Rats

Species		Saline (Control)	Fr-1 (3.0 mg/rat)	Fr-2 (3.0 mg/rat)	Fr-3 (3.0 mg/rat)
Ram	FSH	2.34 ± 0.18 (6)	2.08 ± 0.08 (4)	2.18 ± 0.15 (5)	1.32 ± 0.7* (7)
	LH	1.92 ± 0.10 (6)	1.46 ± 0.12† (5)	2.07 ± 0.19 (5)	1.97 ± 0.08 (5)
Human	FSH	2.69 ± 0.07 (6)	2.65 ± 0.07 (5)	2.71 ± 0.08 (6)	1.94 ± 0.04* (6)
	LH	1.87 ± 0.05 (6)	1.37 ± 0.07† (5)	1.83 ± 0.08 (6)	1.83 ± 0.07 (6)
Rat	FSH	2.69 ± 0.05 (6)	2.73 ± 0.18 (5)	2.75 ± 0.05 (6)	1.99 ± 0.05* (5)
	LH	1.87 ± 0.05 (6)	1.17 ± 0.07* (4)	1.88 ± 0.09 (6)	1.84 ± 0.06 (5)
Chicken	FSH	2.32 ± 0.06 (5)	2.39 ± 0.11 (6)	2.32 ± 0.06 (5)	1.71 ± 0.07* (6)
	LH	1.67 ± 0.05 (6)	1.15 ± 0.03* (6)	1.56 ± 0.11 (6)	1.57 ± 0.19 (6)

The numbers in parentheses represent the number of observations. Serum FSH and LH values are μg/ml (Mean ± SE) expressed in terms of NIAMDD-Rat-FSH-RP-1 and NIAMDD-Rat-LH-RP-1, respectively.
* $P < 0.001$.
† $P < 0.05$.

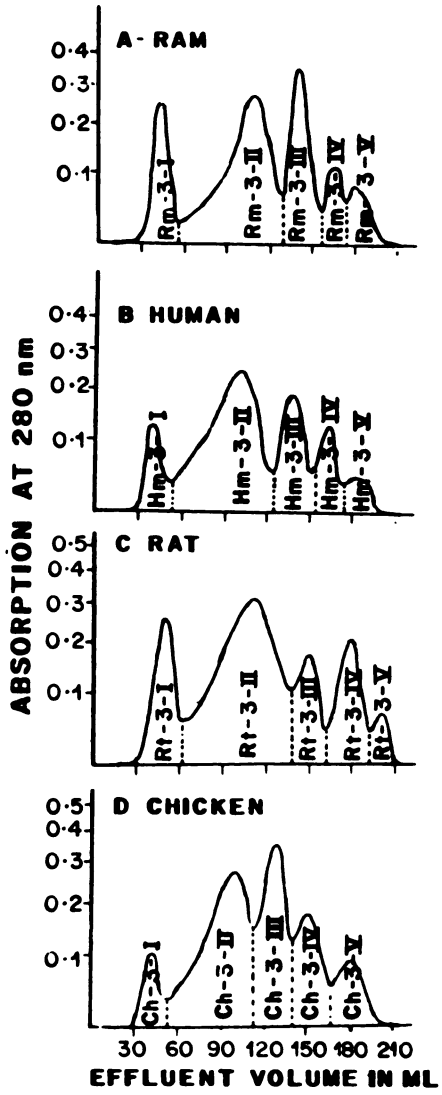

Fig. 2. Elution profile of rechromatography of fractions Rm-3, Hm-3, Rt-3, and Ch-3 on Sephadex G-25 column (2.5 × 38 cm). The elution buffer was 0.01M phosphate buffer, *p*H 7.4. The flow rate was 18 ml/hr, and the fraction volume was 3 ml.

chimont et al (1977) reported that the low-molecular-weight (5000 daltons) species was lost if proteins from rete testis fluid were precipitated with ethanol as an initial step in purification. Davies et al (1978) observed that the large-molecular-weight form (90,000 daltons) can easily be converted into the one with low molecular weight (5000 daltons) with the use of urea. Franchimont et al (1975a) and Thakur et al (1978), using ethanol precipitation as the first step in their purification schemes, have assigned a molecular weight of about 19,000 daltons to their inhibin preparations isolated from bull and human seminal plasma, respectively. Inhibin isolated from bull spermatozoa appears to be a peptide of less than 10,000 daltons (Lugaro et al, 1973, 1974). Lee et al (1977) found inhibin activity to be associated with two fractions of bovine testicular extract, one of molecular weight between 15,000 and 100,000 daltons and the other of 500 to 3000 daltons. Shashidharamurthy et al (1979) have isolated an inhibin preparation with a molecular weight of 20,000 daltons from ram testes. Their method of isolation includes heating of the testicular homogenate at 60 C to remove some of the inert proteins (as coagulum).

The discrepancies between these observations have been attributed either to the existence of multiple forms of inhibin or to its association with carrier proteins (Franchimont et al, 1978; Davies et al, 1978). In view of their own experience, the authors strongly feel that the isolation methodology is also a major contributing factor in determing the molecular size of the isolated inhibin preparation. The use of ethanol and heat may lead to the isolation of larger molecular species, while more gentle conditions, such as have been used here, may

TABLE 2. Inhibin Activity of Fractions Obtained by Rechromatography of Rm-3, Hm-3, Rt-3 and Ch-3 on Sephadex G-25

Treatment	Dose/Rat (mg)	LH μg/ml (Mean ± SE)	FSH μg/ml (Mean ± SE)	Percent Inhibition in FSH Levels	Mean Relative Potency	Fiducial Limits at 95% Confidence Limits
Saline		1.67 ± 0.06 (12)	2.39 ± 0.07 (11)			
Rm-3-II	0.25	1.66 ± 0.08 (5)	1.89 ± 0.05* (5)	20		
	0.50	1.66 ± 0.12 (5)	1.56 ± 0.09* (5)	35		
	1.00	1.56 ± 0.10 (5)	1.34 ± 0.06* (5)	44		
Hm-3-II	0.25	1.57 ± 0.10 (6)	1.45 ± 0.06* (6)	39		
	0.50	1.52 ± 0.12 (6)	1.34 ± 0.04* (6)	44	1.75	1.29–2.59
Rt-3-II	0.25	1.52 ± 0.08 (6)	1.59 ± 0.07* (6)	33		
	0.50	1.53 ± 0.10 (6)	1.32 ± 0.05* (6)	45	1.44	1.06–2.06
Ch-3-II	0.25	1.61 ± 0.09 (3)	1.95 ± 0.07† (6)	18		
	0.50	1.60 ± 0.08 (6)	1.57 ± 0.07* (6)	34	0.65	0.22–1.00

The numbers in parentheses represent the number of observations. Serum LH and FSH are expressed in terms of NIAMDD-Rat-LH-RP-1 and NIAMDD-Rat-FSH-RP-1, respectively.
* $P < 0.001$.
† $P < 0.01$.

permit its isolation as a low-molecular-weight peptide, which the authors feel is its native form.

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