Characterization of a polypeptide from human seminal plasma with inhibin (inhibition of FSH secretion)-like activity

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A polypeptide in preparations of 'large' form (M_r ~14000) material with inhibin-like activity (inhibiting FSH secretion) has been isolated from human seminal plasma. Its amino acid composition, cleavage pattern with CNBr, N-terminal sequence, and properties on reverse-phase high-performance liquid chromatography establish this inhibin-like preparation to be homogeneous. The polypeptide contains close to 130 residues, has a free N-terminal serine residue, a methionine residue in position 19, and a dibasic structure (Arg–Lys) in positions 16–17.

Seminal plasma *Feed-back control* *Amino acid sequence*
High-performance liquid chromatography

1. INTRODUCTION

The secretion of follicle stimulating hormone (FSH) is not regulated entirely by the negative feed-back from gonadal steroids. The steroids can suppress FSH secretion, but they would then also affect secretion of luteinizing hormone (LH). A testicular factor (non-steroidal, water-soluble peptide) has been implicated in the negative feed-back control of FSH secretion and has been named inhibin [1]. Several peptides with inhibin-like activity, but differing in size and purification pattern, depending on organ and species, have been detected from gonads and their secretions (cf. reviews [2–4]). A high-M_r form (M_r ~14000) from human seminal plasma [5] has been reported, as well as low-M_r forms (M_r < 2000) in sheep testes and ovaries [6,7]. The purification and general characterization of the polypeptide corresponding to the large form are described, together with the N-terminal amino acid sequence of the peptide, giving for the first time any structural information on a molecule with inhibin-like activity.

2. MATERIALS AND METHODS

2.1. *Polypeptides*

Large-form inhibin-like polypeptide was prepared as in [5] with some modifications. Briefly, the procedure is as follows: The active material was precipitated from pooled seminal plasma using 4 vols of ethanol. The precipitate was dissolved in sodium acetate buffer (pH 4.0) and loaded on a Sephadex G-100 column (90 × 2.5 cm), using the acetate buffer for elution. The fractions enriched in inhibin-like activity (detectable by bioassay, cf. below) were further purified on a DEAE–Sephadex A-50 clumn (60 × 1.5 cm) using step-wise elution with NaCl (0.02–0.05–0.1 M) in 0.05 M Tris (pH 7.2). The material eluted with 0.05 M NaCl gave a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) at pH 8.3 in Tris–glycine buffer, as in [3].

Reverse-phase high-performance liquid chromatography (RP–HPLC) was carried out using a Waters system with a μBondapak C18 col-

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umn in 0.1% trifluoroacetic acid with an acetonitrile gradient for elution as in [8].

2.2. *Bioassays*

The biological activity of inhibin preparations was measured by the specific inhibition of FSH release in adult male rats and by in vitro incubation with rat pituitaries [9]. The purified fractions already gave positive reactions in these assays at amounts corresponding to about 100 ng and 10 ng polypeptide, respectively. These values are several-fold lower than those with other preparations reported in [9,10], suggesting high biological potency and purity of the present inhibin-like material.

2.3. *Structural analysis*

Carboxymethylation of the polypeptide (140 nmol) with ^{14}C-labelled iodoacetate was carried out at pH 8.1 n 8 M urea, 0.1 M Tris, 2 mM EDTA (1.5 ml) after reduction with dithiothreitol (7.5 μmol, 37°C, 2 h) by reaction with iodo[2-^{14}C]acetate (22.5 μmol, 37°C, 1 h). Subsequently, reagents were removed by extensive dialysis in Spectrapor 3 tubing against water. Cleavage with CNBr was performed in 70% formic acid as in [11]. Chymotrypsin was used in 0.1 M ammonium bicarbonate for 4 h at 37°C at an enzyme:inhibin CNBr-fragment ratio of 1:10. Amino acid compositions were determined with a Beckman 121M amino acid analyzer after hydrolysis in 6 M HCl, 0.5% phenol, at 110°C for 24 h in evacuated tubes. Liquid phase sequencer degradations were performed in a Beckman 890D instrument in the presence of pre-cycled polybrene [12]. Phenylthiohydantoin derivatives were identified by high-performance liquid chromatography (HPLC) [13].

3. RESULTS

3.1. *High-M_r form of inhibin-like material*

A preparation containing the large form of inhibin-like material was obtained from seminal plasma by ethanol fractionation, exclusion chromatography on Sephadex G-100, and ion exchange chromatography on DEAE–Sephadex [5]. This material, apparently homogeneous on SDS–PAGE [3], was submitted to RP–HPLC as described in section 2. The result is shown in fig.1, further indicating that the preparation of large form inhibin-like activity contains only one major peptide. This peptide is already largely pure before the step of HPLC.

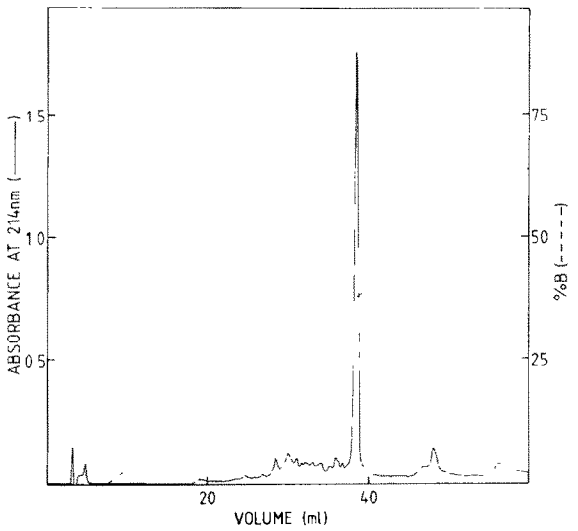

Fig.1. RP–HPLC of the large-form preparation of inhibin-like activity on μBondapak C18 in 0.1% trifluoroacetic acid and a gradient (% B) of acetonitrile (flow 1.5 ml/min).

3.2. *Characterization*

The polypeptide purified was ^{14}C-carboxymethylated with ^{14}C-labelled iodoacetate in 8 M urea after reduction as described in section 2. The total composition of the carboxymethylated polypeptide is given in table 1. Acceptable amino acid ratios are obtained with a minimum polypeptide size of about 130 residues. This is in excellent agreement with the M_r of 14000 estimated by SDS–PAGE against cytochrome *c* and myoglobin as standards.

The composition suggests that the polypeptide contains one methionine residue (table 1). One aliquot (70 nmol) was therefore cleaved with CNBr in 70% formic acid. Subsequent exclusion chromatography on Sephadex G-50 in 30% acetic acid gave two peptide fractions as expected, fraction 1 eluting close to the void volume of the column, and fraction 2 eluting much later but still well separated from low-M_r UV-absorbing material derived from the reagents used.

Table 1

Amino acid compositions of the polypeptide in large-form preparations of inhibin-like activity, its CNBr fragments, and the constituent chymotryptic peptides of the N-terminal CNBr fragment

Peptide	Whole polypeptide		CNBr 1		CNBr 2		CNBr 2A		CNBr 2B	
Cys(Cm)	9.6	(10)	6.3	(8)	1.3	(2)	1.0	(1)	0.8	(1)
Asx	16.9	(17)	13.7	(15)	2.0	(2)	–	–	1.9	(2)
Thr	9.5	(10)	8.4	(9)	1.1	(1)	–	–	0.9	(1)
Ser	13.7	(13)	9.6	(11)	2.0	(2)	1.0	(1)	1.0	(1)
Glx	20.1	(18)	15.1	(17)	1.2	(1)	–	–	1.2	(1)
Pro	6.6	(7)	5.0	(5)	2.1	(2)	–	–	2.0	(2)
Gly	11.2	(10)	8.4	(8)	2.2	(2)	–	–	2.1	(2)
Ala	3.9	(4)	3.7	(4)	–	–	–	–	–	–
Val	6.9	(7)	6.3	(6)	1.0	(1)	–	–	1.0	(1)
Met	0.8	(1)	–	–	0.6	(1)	–	–	+	(1)
Ile	6.1	(6)	5.2	(5)	1.0	(1)	–	–	0.9	(1)
Leu	5.2	(5)	4.4	(5)	–	–	–	–	–	–
Tyr	3.9	(5)	3.8	(4)	0.9	(1)	1.1	(1)	–	–
Phe	3.0	(3)	2.0	(2)	1.0	(1)	–	–	0.9	(1)
Trp	1.4	(1)	+	(1)	–	–	–	–	–	–
Lys	11.2	(11)	9.9	(10)	1.0	(1)	–	–	1.1	(1)
His	2.5	(2)	1.4	(2)	–	–	–	–	–	–
Arg	3.2	(3)	1.7	(2)	0.8	(1)	–	–	0.9	(1)
Sum	~133		~114		19		3		16	

Intact carboxymethylated polypeptide (left column), the two CNBr fragments (CNBr 1 and CNBr 2), and the two chymotryptic peptides (CNBr 2A and CNBr 2B) were hydrolyzed with 6 M HCl, 0.5% phenol, for 24 h at 110°C; intact inhibin also with 4 M methanesulfonic acid, 0.2% 3-(2-aminoethyl)indole for the tryptophan analysis. Analytical values are molar ratios, with assumed integers within parentheses. Original Met in CNBr 2 and CNBr 2B was recovered as homoserine + homoserine lactone

Fractions 1 and 2 both corresponded to pure fragments as shown by sequence analysis (below). The compositions of both fragments are given in table 1, and the two peptides appear to account for all residues present in the whole polypeptide. Furthermore, already the compositions show one of the fragments (fragment CNBr 2) to have, and one of them (fragment CNBr 1) to lack homoserine (table 1), as expected for two major fragments from a parent polypeptide with one methionine residue.

The whole polypeptide was submitted to direct liquid phase sequencer analysis as shown in table 2. Similarly, fragments CNBr 1 and CNBr 2 were also separately degraded. The residue in position 18 was not fully identified from the sequencer degradations alone, and the composition of CNBr 2 was low in carboxymethylcysteine (table 1) suggested for that position (table 2). Therefore, one aliquot of CNBr 2 was cleaved with chymotrypsin. The two peptides obtained were separated by RP–HPLC and both were found to contain one residue of carboxymethylcysteine (also shown in table 1), establishing the assignment in position 18.

As shown in table 2, the results from the sequencer degradations are consistent with the conclusions from the compositions in showing fragment CNBr 2 to be derived from the N-terminal region of the polypeptide and fragment CNBr 1 to be derived from the remaining part by cleavage with CNBr at a methionine residue in position 19.

Table 2

N-terminal structure of the polypeptide in large-form preparations of inhibin-like activity as determined by liquid-phase sequencer analysis of the intact peptide, the N-terminal CNBr fragment from the ^{14}C-carboxymethylated protein (CNBr 2 from the elution order on exclusion chromatography), and the second CNBr fragment (CNBr 1)

Inhibin structure		Analytical results		
Position	Residue deduced	Intact peptide	CNBr 2	CNBR 1
1	Ser	Ser H	Ser H	
2	Cys	–	CysCm H R	
3	Tyr	Tyr H 25	Tyr H 4	
4	Phe	Phe H 25	Phe H 5	
5	Ile	Ile H 30	Ile H 5	
6	Pro	Pro H	Pro H	
7	Asn	Asn H	Asn H	
8	Glu	Glu H	Glu H	
9	Gly	Gly H	Gly H	
10	Val	Val H 25	Val H 3	
11	Pro	Pro H	Pro H	
12	Gly	Gly H	Gly H	
13	Asp	Asp H	Asp H	
14	Ser	Ser H	Ser H	
15	Thr	Thr H	Thr H	
16	Arg	Arg H	Arg H	
17	Lys	Lys H	Lys H	
18	Cys	–	(CysCm H R)	
19	Met	Met H 20		
20	Asp	Asp H		Asp H
21	Leu	Leu H 20		Leu H 3
22	Lys	Lys H		Lys/Phe H 2
23	Gly	Gly H		Gly H
24	Asn	Asn H		Asn H
25	Lys	Lys H		Lys/Phe H 2
26	His	–		His H
27	Pro	Pro H		Pro H
28	Ile	Ile H 15		
29	Asn	Asn H		
30	Ser	Ser H		

Residue identifications were by HPLC (H), and where applicable by radioactivity (R) for the ^{14}C-carboxymethylated fragments. Values give nmol recovered of stable thiohydantoin derivatives from degradations of 5–40 nmol

4. DISCUSSION

4.1. *Homogeneity*

The consistent results from sequence analysis, CNBr cleavage and total compositions fully confirm that the present preparation with inhibin-like activity is essentially homogeneous in relation to a major polypeptide with N-terminal serine and a total of about 130 residues. In addition, the N-terminal structure was established. The single band upon gel electrophoresis and the single peak upon RP–HPLC (fig.1) also support purity of the starting material. It may therefore be concluded that a single polypeptide is the major component of large form preparations with inhibin-like activity from seminal plasma.

4.2. *Structural characteristics*

The polypeptide in seminal plasma large-form inhibin-like preparations has a single methionine residue positioned in the N-terminal part. The N-terminus is formed by a serine residue. Such a residue is often blocked by acetylation in intact proteins [14], but this is not the case in the present large-form inhibin-like material, and N-terminal serine is also found not to be blocked in another biologically active peptide such as ACTH.

Since low-M_r forms of polypeptides with inhibin-like activity have been reported in preparations from other organs [6,7], possible cleavage sites in the present large form are of particular interest. If the large form can give rise to the smaller forms, cleavages at dibasic structures would be especially expected from the structures of known hormonal pro-forms [15] and from the apparent presence of restricted sequences in relation to such possible cleavage sites [16]. Regarding dibasic structures, it may be noticed that the polypeptide now characterized has an Arg–Lys sequence at positions 16–17, although additional such structures may of course also occur elsewhere in the molecule.

The structure determined has apparently not been reported before in biologically active peptides or other polypeptides. Consequently, the polypeptide corresponding to the major component of large-form inhibin-like material has been purified, and its structural characteristics have been determined.

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