Analysis of an inhibin preparation reveals apparent identity between a peptide with inhibin-like activity and a sperm-coating antigen

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The amino acid sequence of a large form of inhibin-like peptide in human seminal plasma was determined, and compared with structures reported for similar inhibin preparations and a seminal plasma globulin. The data confirm and correlate previous reports on this form of inhibin-like peptide. The structural comparisons further suggest that the peptide is closely similar to or possibly identical to a sperm-coating antigen reported to be synthesized from prostatic epithelium. This may correlate with non-gonadal origins of inhibin-like material and will help to elucidate the biological roles of inhibin(s).

Inhibin *Seminal plasma* *Primary structure* *Structural comparison*

1. INTRODUCTION

Inhibin is the name given to the postulated hormone that has been implicated in the selective suppression of FSH secretion [1]. The corresponding peptide is of presumed gonadal origin and considered to decrease the hypophyseal release of FSH but not that of LH. It is therefore more selective than the gonadal steroid hormones which influence both FSH and LH secretion. Further characterizations of this peptide(s) have been complicated by difficulties with interpretations of bioassays, and there is not complete agreement even on the existence of the peptide(s). Furthermore, several peptides, differing in size and other properties have been reported to have inhibin-like activity (cf. [2,3]). Finally, similar peptides have been suggested to occur in gastric secretions [4], in blood plasma of gonadectomized individuals [5], and in other organs, raising doubts concerning the gonadal origin or functional specificity of inhibin activity.

Recently, the situation was made clearer by several structural studies on inhibin-like peptides [2,3,6,7]. Thus, a high-molecular-weight form was first purified and its N-terminal structure characterized [2]. A completely different structure with lower molecular weight was similarly reported [3]. A study on sperm antigen analysis [6], which was not concerned with inhibin activity, revealed a structure related to high-molecular-mass, inhibin-like peptide; and the structure of the latter inhibin preparation was reported, showing apparent bioactivity of a C-terminal fragment [7], possibly limited structural similarity to another seminal peptide [8], and probable pro-form cleavages [7].

All these reports provide new and clearly defined standards for further inhibin correlations. Here, a separate analysis independent of [7], for the high-molecular-mass inhibin-like peptide [2] is reported. This allows comparisons between 3 preparations ([2,7], this work), correlating all data. Comparisons also suggest further conclusions on the origin(s) and biological function(s) of these polypeptides, as well as suggesting possible future tests.

2. MATERIALS AND METHODS

Large-form inhibin from human seminal plasma was prepared by ethanol precipitation and subse-

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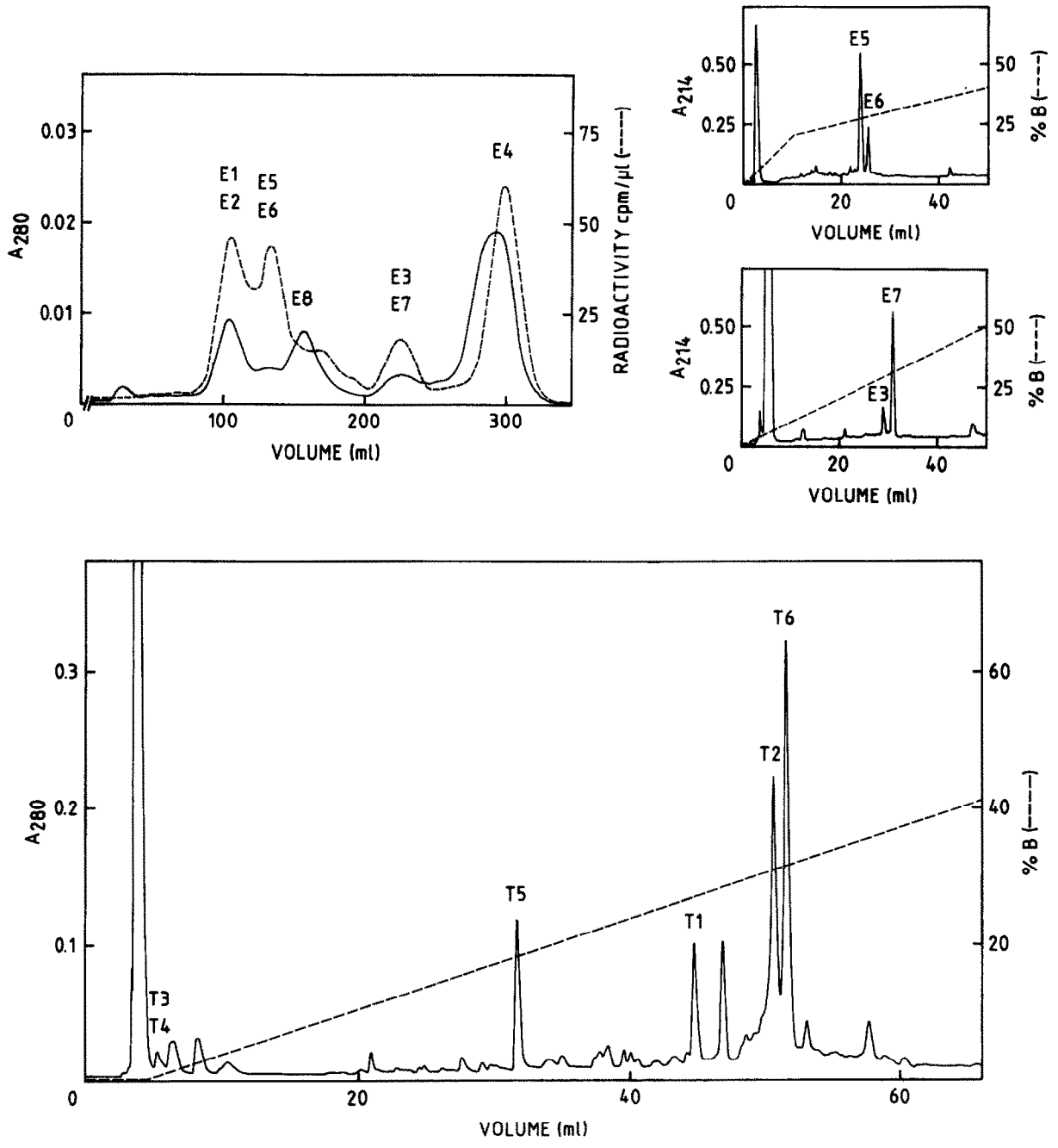

Fig.1. Purification of enzymatic peptides from the carboxymethylated large form of the inhibin-like peptide (peptide nomenclature as in fig.2). (A) Left: Sephadex G-50 exclusion chromatography (1.5 × 200 cm; 30% acetic acid) of the digest with Glu-specific Staphylococcal protease. Positions of all E-peptides shown in fig.2 are indicated. Right: Purification by reverse-phase HPLC (µBondapak C18; gradient of acetonitrile, %B, in 0.1% trifluoroacetic acid) of the fragments corresponding to the E5/E6 and E3/E7 mixtures, respectively. (B) Purification of the tryptic digest by direct reverse-phase HPLC as in A.

quent column chromatographies as previously described [2]. The peptide was reduced, carboxymethylated with ^{14}C-labelled iodoacetate [2], and subjected to direct sequencer analysis, as well as for separate cleavages with CNBr [2], trypsin, Staphylococcal Glu-specific protease, and clostripain. The enzymatic digestions were carried out for 4 h at 37°C, with inhibin amounts of 20–100 nM in 200–500 μl of 0.1–0.2 M ammonium bicarbonate and 5–50 μg enzyme (least for trypsin, most for clostripain). Digests were purified by reverse-phase high performance liquid chromatography (HPLC), using linear gradients of acetonitrile in 0.1% trifluoroacetic acid, as described [9]. After the treatment with Staphylococcal protease, digests were prefractionated by exclusion chromatography on Sephadex G-50 in 30% acetic acid [9]. Amino acid compositions were determined with a Beckman 121M analyzer after hydrolysis for 24 h at 110°C with 6 M HCl/0.5% phenol. Liquid phase sequencer analyses were performed in a Beckman 890D instrument in the presence of glycine-precycled Polybrene [10] and using a 0.1 M Quadrol peptide program; phenylthiohydantoins were analyzed by reverse-phase HPLC [11]. Manual sequence degradations were carried out by the DABITC double coupling method [12], utilizing by-products to assist identifications by thin-layer chromatography [13].

3. RESULTS

The primary structure of the inhibin-like peptide was determined by liquid-phase sequencer analysis of the whole carboxymethylated peptide, combined with sequencer-assisted and manual degradations of the fragments obtained after separate treatments with CNBr, trypsin, Staphylococcal Glu-specific protease, and clostripain, respectively. All peptides were purified by

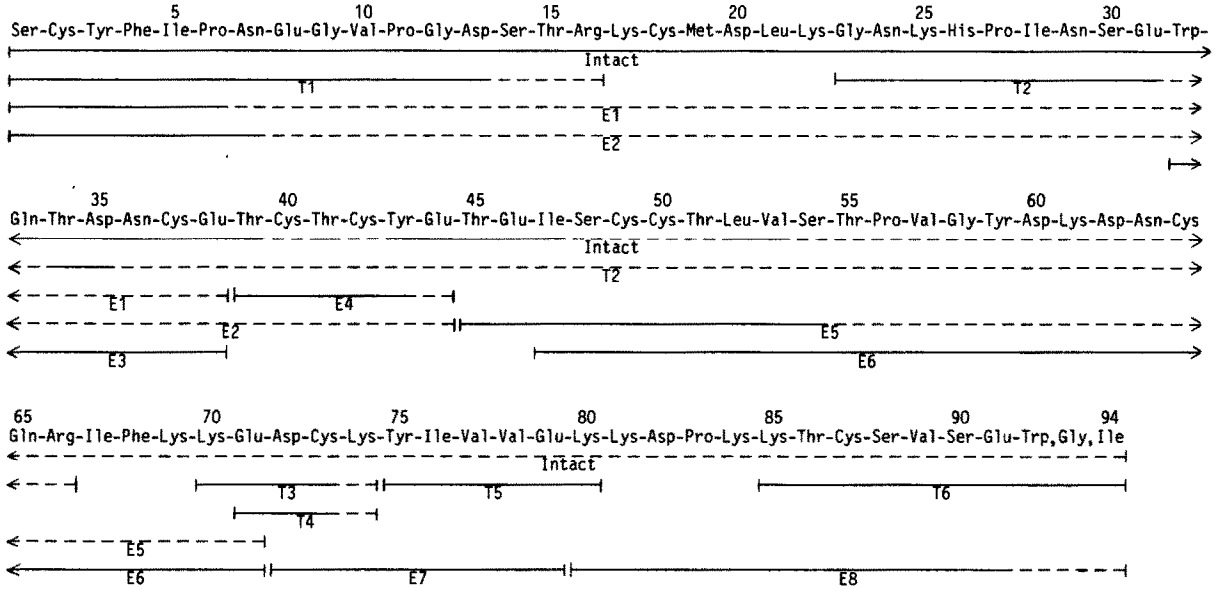

Fig.2. Primary structure of the inhibin-like peptide analyzed, together with positions of fragments. Cys residues analyzed as Cys(Cm) after reduction and carboxymethylation, but are likely to occur as half-cystines in the native structure (cf. [7,8]). Peptides derived from treatments with trypsin are denoted by T, with Glu-specific Staphylococcal protease by E, and with CNBr by CN. Numbers give sequential order of all fragments within each set of peptides. Regions of peptides identified by Edman degradation (liquid phase sequencer or DABITC degradations) are given by solid lines, regions proven by compositions only by dotted lines. Low sequence yields of the C-terminal T6 and E8 residues are indicated by commas between residues 92–94. Peptides were purified as shown in fig.1, relevant compositions are given in table 1. In addition to the fragments shown, the two CNBr fragments were purified as given in [2] and 3 peptides after clostripain digestion (Arg-cleavage) were isolated.

reverse-phase HPLC and/or Sephadex G-50 exclusion chromatography.

Purifications of the digests with trypsin and the Staphylococcal protease are shown in fig.1, and analytical results are summarized in fig.2. Compositions of peptides relevant to the deduction of the structure are given in table 1.

4. DISCUSSION

4.1. *Structure analysis*

Most peptides were analyzed with unequivocal results, as shown in fig.2, but three minor problems were encountered and required further consideration. One was the approach to analysis of the middle region (after position 40) by direct sequencer analysis of the intact peptide or the C-terminal CNBr fragment. Thus, such analysis did not proceed as far as anticipated because of successive increases in residue carry-over, which appeared to increase at each of 5 Glu/Gln positions between residues 31–46, successively obscuring interpretation of direct analyses both of the intact peptide and of the large CNBr fragment. Consequently, the middle region had to be established by isolation and analysis of smaller peptides, obtained by enzymatic cleavages, as indicated (E3–E6, fig.2). Here, as well as in the C-terminal region, partial cleavages, yielding overlapping peptides in lower yield were also encountered (E5/E6 and T3/T4). The T3/T4 doublet of peptides was incompletely resolved upon high-performance liquid chromatography and was therefore degraded as an unresolved mixture, which was sufficient to establish the E6/E7 overlap.

Table 1

Amino acid compositions of fragments used to deduce the structure of the inhibin-like peptide

Peptide	T6	E6	E7	E8	CN2	Sum (CN1+CN2)
Cys (Cm)	5.5 (6)	2.7 (3)	1.0 (1)	1.0 (1)	7.0 (8)	8.9 (10)
Asx	6.4 (7)	3.4 (3)	1.1 (1)	1.2 (1)	10.5 (10)	12.5 (12)
Thr	5.3 (6)	2.1 (2)	– –	1.0 (1)	6.4 (7)	7.5 (8)
Ser	3.1 (3)	2.2 (2)	– –	2.2 (2)	5.9 (5)	7.9 (7)
Glx	6.2 (6)	2.4 (2)	1.2 (1)	1.2 (1)	10.2 (9)	11.4 (10)
Pro	1.9 (2)	0.9 (1)	– –	0.9 (1)	2.9 (3)	5.0 (5)
Gly	2.3 (2)	1.1 (1)	– –	0.9 (1)	3.4 (3)	5.6 (5)
Ala	– –	– –	– –	– –	0.4 –	0.4 –
Val	2.0 (2)	2.1 (2)	1.9 (2)	1.0 (1)	4.4 (5)	5.4 (6)
Met	– –	– –	– –	– –	– –	0.6 (1)
Ile	2.0 (2)	1.9 (2)	1.0 (1)	0.7 (1)	4.5 (5)	5.5 (6)
Leu	1.1 (1)	1.1 (1)	– –	– –	2.1 (2)	2.1 (2)
Tyr	1.9 (2)	1.0 (1)	0.9 (1)	– –	2.7 (3)	3.6 (4)
Phe	– –	0.9 (1)	– –	– –	1.0 (1)	2.0 (2)
Trp	+ (1)	– –	– –	+ (1)	1.9 (2)	1.9 (2)
Lys	1.9 (2)	3.0 (3)	1.1 (1)	3.8 (4)	10.2 (10)	11.2 (11)
His	0.8 (1)	– –	– –	– –	1.0 (1)	1.0 (1)
Arg	1.0 (1)	0.9 (1)	– –	– –	1.1 (1)	1.9 (2)
Sum	44	25	8	15	75	94

Peptide nomenclature and positions as in fig.2; purifications as in fig.1. Analytical values are molar ratios from acid hydrolysis (uncorrected for impurities, destruction, or slow release), with integers from the sequence determination within parentheses. Data for CN1 have been reported before [2], with Met analyzed as Hsl + Hse; Trp in CN2 determined after hydrolysis with methane sulfonic acid. Last column gives the agreement for fragments corresponding to the whole original peptide

The second problem encountered was the C-terminal structure. The last 3 residues were not completely reliably identified as phenylthiohydantoin derivatives. Thus, the yield of a tryptophan derivative corresponding to residue 92 was extremely low and the order of residues 93 and 94 was hardly distinguishable although PTH-Gly increased before PTH-Ile on degradation of T6. The presence of tryptophan in this region was also supported by the UV absorption of T6 (fig.1) and by direct tryptophan analysis after methanesulfonic acid hydrolysis of the whole peptide. The C-terminal order in fig.2 is given within commas to denote the difficulties with these interpretations.

The final problem was in purity of the starting material and in migration of the intact peptide upon sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). Thus, contaminants in the starting material increased the values for Ala initially deduced from acid hydrolysis of the whole peptide, and the electrophoretic migration suggested a longer peptide [2]. Re-purification of CN2 clearly showed the absence of Ala. Furthermore, the sum of residues by acid hydrolysis (last column in table 1) and by sequence degradations (fig.2) agree and prove a size 10–20% smaller than at first seemed likely from the electrophoretic migration.

Consequently, the complete structure is concluded to be as given in table 2. This result is also in agreement with an independent analysis [7] where problems of a similar nature were encountered, proving complete identity between these inhibin preparations.

4.2. *Inhibin as a sperm-coating antigen*

The large-form inhibin structure is almost identical to the structure of a sperm-coating antigen reported in [6]. In the preliminary report of the sperm-coating antigen [6] only minor deviations exist, at two internal positions (39 and 40) and at the last two C-terminal positions. It may be possible that the deviations in these two regions reflect racial or other differences. However, it also seems possible that these deviations are only apparent, especially if the difficulties in the analysis of 'sperm-coating antigen' were similar to those encountered in the analysis of 'inhibin' preparations (cf. above). In any event, the analyses given in 3 reports ([6,7], this work), now combine clearly to establish that the sperm-coating antigen, of importance in detecting seminal stain in forensic medicine [6] and of occurrence as a β-globulin in human seminal plasma [6], is fully or partly identical to the 'high-molecular-mass form of inhibin' [2] or 'β-inhibin' [7].

This fact, derived from structural studies, is also relevant to two other previously unexplained observations on inhibin-like peptides. Thus, some reports suggest that such peptides, although of presumed gonadal origin, may also occur in other organs [4,14] or in gonadectomized individuals [5]. This is consistent with identity/similarity with seminal β-globulin, since the latter has an extragonadal origin, being synthesized by prostatic epithelial cells and found in increased concentrations in serum of patients with prostatic cancer [6]. In fact, prostatic origin of inhibin-like peptides has also been independently indicated [14]. Furthermore, observations on recoveries during purification of present inhibin preparations suggest that the amount of inhibin is larger than might normally be expected for a peptide with only hormonal function; the amount of the inhibin-like peptide is therefore consistent with it being a seminal plasma β-globulin [6].

Regarding function, the occurrence and amount should not be taken to disqualify the present peptide from being an inhibin-like hormone, since some hormones occur in considerable quantities even in relation to other factors within a single family (like, for example, insulin-like growth factor 1, IGF-1, which is much more abundant in blood plasma [15] than the structurally related insulin, presumably because most of IGF in plasma is associated with a binding protein [16]).

4.3. *Future tests*

The combined results on the structural characterizations of inhibin molecules, and the finding of peptides identical or nearly identical to previously known seminal plasma proteins/sperm antigens, offer excellent possibilities for establishing inhibin-like bioactivities and relationships, as also pointed out in [7]. Already now, the similarity/identity with a seminal β-globulin of prostatic origin [6] suggests that the present peptide may also have a non-gonadal origin, consistent with previous immunological studies [4,5]. It therefore appears important to repeat these studies with antibodies

against defined and synthetic replicates of the structure to establish the origin(s) of inhibin.

The finding of other inhibin-like peptides [3] and of active fragments of the present molecule [7], as well as the possible similarities with parts of an enzyme [3] or with gastrointestinal-brain prohormones [7] offer further opportunities for correlations with origin and function. Finally, the availability of an established structure will allow synthesis of oligonucleotide probes. For all these purposes, it is important to have complete agreement between different preparations ([2,7], this work) and regarding assignment of inhibin to a known seminal protein of prostatic origin [6]. Agreement is now evident from the structural comparisons.

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