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HIGH MOLECULAR WEIGHT FORMS OF RELAXIN IN PREGNANT SOW OVARIES

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SUMMARY

The existence of a prohormone for relaxin has been investigated by purification from extracts of pregnant sow ovaries. Radioimmunoassay of column fractions from large scale extraction of frozen pregnant sow ovaries detected two species of high molecular weight relaxin immunoactivity besides the 6,300 dalton relaxin. On further purification, these two species were resolved into three forms with apparent molecular weights of 19,000, 13,000 and 10,000 daltons respectively. Each was biologically active and could be converted by trypsin to the 6,300 dalton relaxin. They may represent intermediates of relaxin.

INTRODUCTION

Relaxin is a 6,300 dalton peptide hormone first described by Hisaw in 1926 (1). It was purified from pregnant sow ovaries by Sherwood and O'Byrne (2) and its amino acid sequence was recently determined by two groups of investigators (3, 4, 5). The striking structural similarity to insulin (6, 7, 8) suggests the existence of a precursor molecule for relaxin.

High molecular weight forms of many hormones have been demonstrated in both plasma and tissue extracts (9, 10). Earlier work in this laboratory suggested the possible existence of a prohormone of relaxin in plasma and tissue extracts (11, 12). Recently, evidence of a "prorelaxin" in a crude preparation of porcine relaxin (NIH-R-P1) has been demonstrated (13). However, these high molecular weight forms of relaxin have not been well characterized. In this communication, we report the isolation and characterization of three high molecular weight forms of relaxin from frozen pregnant sow ovaries.

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MATERIALS AND METHODS

Isolation: Frozen pregnant sow ovaries (Farmer John, Los Angeles) were extracted with acetone-HCl by the method of Sherwood and O'Byrne (2). The crude extract was then chromatographed at 4°C on a 6.5 x 114 cm column of Sephadex G.50 (fine) using 0.2 M ammonium acetate buffer, pH 6.8 as eluant. Flow rate was 92 ml/hr. Fractions of 10 ml were collected. Further chromatography was carried out on a 1.5 x 85 cm column of Sephadex G.50 (superfine). The column was equilibrated and eluted with 0.08 M ammonium acetate, pH 5.5 at a flow rate of 8 ml/hr. Fractions of 1 ml were collected. The fractionation steps were monitored by radioimmunoassay. The column was calibrated with chymotrypsinogen A, soybean trypsin inhibitor, myoglobin, ribonuclease A, pancreatic trypsin inhibitor, porcine relaxin and glucagon.

Tryptic digest: Sample (500 ug) was dissolved in 300 ul of PBS containing 10 mg $CaCl_2$/100 ml and warmed up to 37°C. TPCK-trypsin (5 ug in 100 ul same buffer) was then added and the mixture was incubated for 5 min. The digestion was terminated by adding 100 ug of soybean trypsin inhibitor (in 100 ul PBS), followed by 500 ul of buffer A. The mixture was immediately applied to a 1.5 x 85 cm column of Sephadex G.50 (superfine) and eluted with buffer A as described above. For a control, an equal amount of sample was treated with 1 ml of 8 M urea in PBS at room temperature for 24 hrs. prior to gel filtration.

Radioimmunoassay: Highly purified relaxin (fraction CM-a' as designated by Sherwood and O'Byrne (2)) was radioiodinated by the Bolton and Hunter method (14) with slight modification (12). The antiserum used was raised in a New Zealand albino rabbit to the highly purified relaxin (CM-a') by the method of Vaitukaitis et al (15). The radioimmunoassay has been used exactly as described (16).

Bioassay: The *in vitro* method using estrogen-primed rat uterine segments (17) was employed with slight modification (18). Relaxin NIH-R-P1 (442 GPU/ml) was used as standard.

RESULTS AND DISCUSSION

When the HCl-acetone extract of frozen pregnant sow ovaries was chromatographed on a Sephadex G.50 (fine) column, three major peaks were detected by absorbance at 280 nm (Fig. 1). The middle one (pool III) was the biologically active 6,300 dalton relaxin. However, radioimmunoassay of the column fractions revealed two peaks of immunoreactivity (pool I and II) larger than the 6,300 dalton relaxin (pool III). These large relaxin immunoreactives represented 3% of the total immunoreactivities.

Rechromatography of pool I on a smaller Sephadex G.50 (superfine) column gave two peaks as detected by UV absorbance at 280 nm (Fig. 2A). The first

Abbreviations: PBS = phosphate buffered saline (0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.4); TPCK = L-1-tosylamide-2-phenylethyl-chloromethyl ketone; buffer A = PBS containing 0.1% fetal calf serum and 0.02% sodium azide; GPU = guinea pig units.

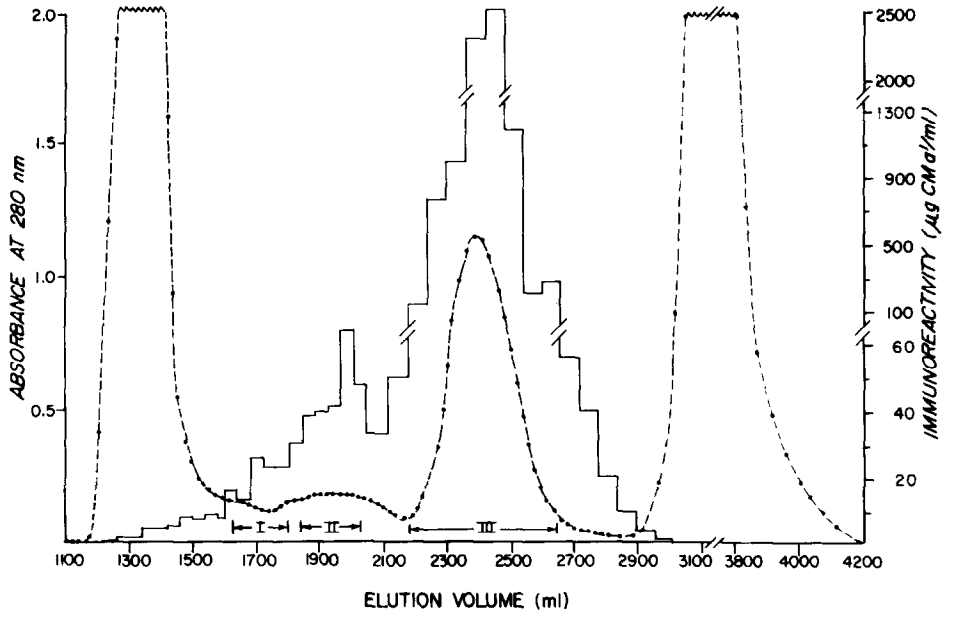

Fig. 1. Gel filtration of a HCl-acetone extract of 1 Kg of pregnant sow ovaries on a 6.5 x 114 cm column of Sephadex G.50 (fine). The column was eluted with 0.2 M ammonium acetate buffer, pH 6.8 at a flow rate of 92 ml/hr. Fractions of 10 ml were collected. Absorbance at 280 nm (●) and immunoreactivity (histogram).

peak (A) appeared at the void volume and was devoid of any immunoreactivity. The second peak (B) contained all the immunoreactivity and had an apparent molecular weight of 19,000 daltons. No significant peak was detected by either UV absorbance or radioimmunoassay at the elution volume of relaxin. Pool II, on the other hand, gave a single absorbance peak, when rechromatographed on the Sephadex G.50 (superfine) column (Fig. 2B). Radioimmunoassay of the column fractions showed three immunoreactive peaks. The first two peaks, namely C and D, had an apparent molecular weight of 13,000 and 10,000 daltons respectively. The third peak (E) corresponded to the elution volume of 6,300 dalton relaxin, and was therefore not studied further.

Incubation of peaks B and C with 8 M urea at room temperature for 24 hrs. prior to rechromatography on a Sephadex G.50 (superfine) column did not change their elution profile (Fig. 3A and 3C). A single major peak of high

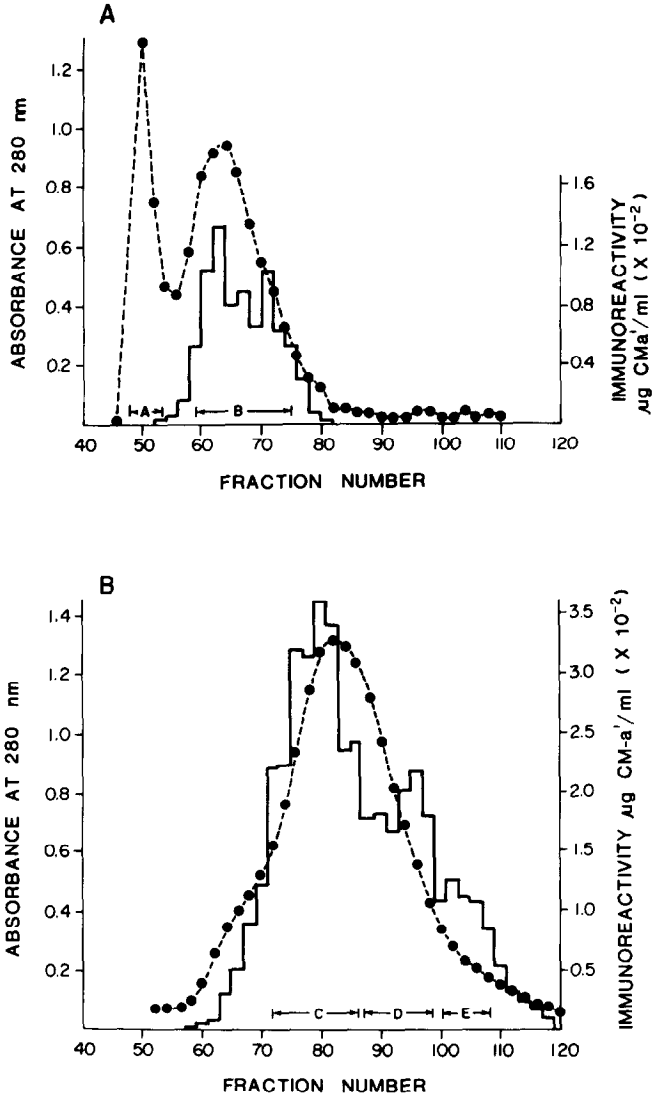

Fig. 2. Rechromatography of (A) pool I and (B) pool II on Sephadex G.50 (superfine). Pools I and II from Fig. 1 were lyophilized. A sample of pool I (40 mg) or of pool II (90 mg) was dissolved in 1 ml of 0.08 M ammonium acetate buffer, pH 5.5 and applied on a 1.5 x 85 cm column of Sephadex G.50 (superfine) eluted with same buffer at a flow rate of 8 ml/hr. Fractions of 1 ml were collected. Alternate fractions were measured for absorbance at 280 nm (●) and immunoreactivity (histogram).

molecular weight was observed even when chromatography was carried out on a Sephadex G.75 column and eluted with buffer containing 7 M urea (data not

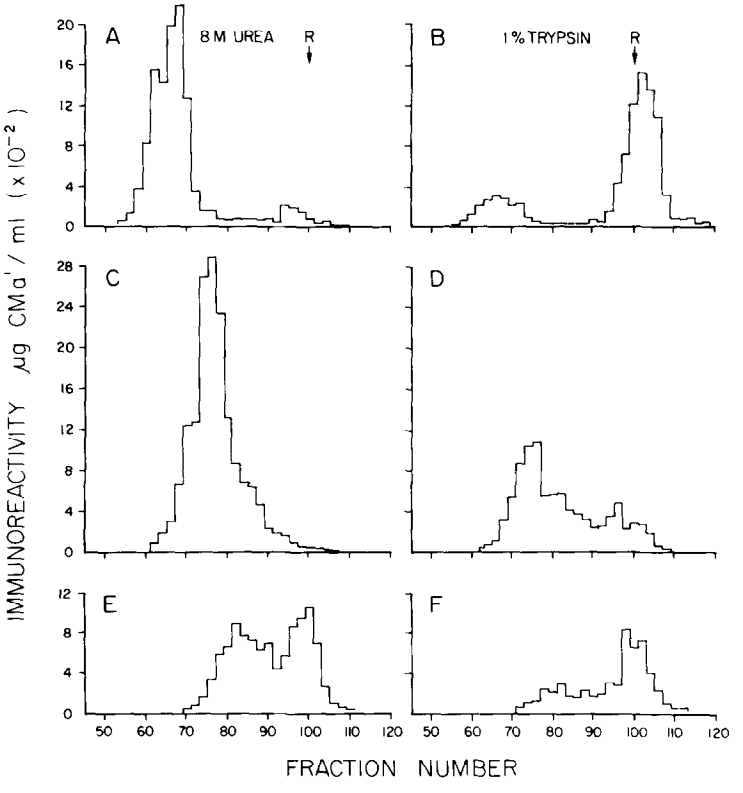

Fig. 3. Rechromatography of (A, B) peak B, (C, D) peak C and (E, F) peak D (from Fig. 2) after treatment with either urea or trypsin. Each sample (500 µg) was treated with urea at room temperature for 24 hrs. (A, C, E) or with 1% (w/w) TPCK-trypsin at 37°C for 5 min. (B, D, F) prior to rechromatography on a 1.5 x 85 cm column of Sephadex G.50 (superfine) eluted with buffer A at a flow rate of 8 ml/hr. Fractions of 1 ml were collected. Aliquots from alternate fractions were taken for radioimmunoassay.

shown). With similar treatment, 60% of the total immunoreactivity of peak D retained its high molecular weight, whereas the other 40% appeared as a component in the elution volume of 6,300 dalton relaxin (Fig. 3E). This may be due to contamination of the peak D by the 6,300 dalton relaxin because of their close molecular weights. Therefore, these high molecular weight forms of relaxin (peaks B, C and D) are unique components and are not nonspecific polymers or aggregates of relaxin with other cellular proteins. Furthermore, it is unlikely that the three high molecular weight forms are interchain disulfide-linked dimer or polymers, as in the case of human placental lactogen

(19) and human growth hormone (20). Relaxin has no free sulfydryl groups (5). All purification steps were carried out at pH 7.4 or below, disulfide interchange would be unlikely to occur.

Incubation of peak B with 1% (w/w) TPCK-trypsin at 37°C for 5 min. converted 66.2% of the original immunoreactivities to a product having the same elution volume of relaxin (Fig. 3B). There was a slight decrease (16.2%) in the total immunoreactivity after trypsin-treatment. Peaks C and D were found to be more sensitive to trypsin. The recovery of total immunoreactivities for peaks C and D after treatment of 1% trypsin at 37°C for 5 min. were 59.1% and 53.0% respectively. Only 14.2% of the original immunoreactivity of peak C was converted to a size of 6,300 daltons (Fig. 3D). There was no net increase of immunoreactivity at the elution volume of 6,300 dalton relaxin when peak D was treated with trypsin (Fig. 3F).

All three species caused inhibition of spontaneous uterine contraction *in vitro* both before and after treatment with 1% trypsin at 37°C for 5 min. (Table I). However, there was no significant increase in biological activity after tryptic digestion. The slopes of the dose-response curves for peaks C and D were not parallel to that of the relaxin standard curve (NIH-R-P1) before tryptic conversion, but became parallel afterwards. On the other hand, the slope of the dose-response curve for peak B was parallel to that of the standard curve, and the biopotency of peak B was significantly higher than those of peaks C and D. This suggests that peak B may be the "true" precursor, and that peaks C and D may be only transient conversion intermediates. The direct conversion of peak B to 6,300 dalton relaxin (Fig. 3B) supported this idea. Although most prohormones are biologically inactive or possess very low activity, procalcitonin (21), big gastrin (22) and high molecular weight forms of ACTH (23) are biologically active. It is not known whether the biological activities of the high molecular weight forms of relaxin reported here are their intrinsic properties or are due to enzymic conversion to the 6,300 dalton form by the uterine segment during bioassay.

Table I. Biopotencies of the three fractions together with the 95% confidence limit (shown in bracket). NIH-R-P1 (442 GPU/mg) was used as the standard.

Molecular weight form	Before tryptic conversion	After tryptic conversion
19,000 (peak B)	1056 (722-1543) GPU/mg	1251 (796-1945) GPU/mg
13,000 (peak C)	NP*	292 (177-486)
10,000 (peak D)	NP	154 (115-203)

*NP = not parallel to standard

The three high molecular weight forms of relaxin seem to be too large for the precursor(s) of a peptide having similar size and structure to insulin, since proinsulin has a molecular weight of only 9,100 daltons (24). The high molecular weights are not due to varying amounts of a carbohydrate moiety as in the case of ACTH (25), because none of the three species were bound to a Con A-Sepharose column (data not shown). It appears that they are not artefacts of the isolation procedure, because they were also detected by fractionation of an acid extract of a fresh 90 day pregnant sow ovary on Sephadex G.50 (superfine) column, followed by radioimmunoassay of the column fractions (data not shown). In addition, two higher molecular weight forms (above 30,000 and 26,000 daltons) were detected in the fresh pregnant sow ovary, but not in the frozen ones, possibly due to autolysis occurring in the latter. The highest molecular weight form was in the same range of 42,000 daltons reported by Frieden and Yeh (13) for a "prorelaxin" from a crude preparation of relaxin (NIH-R-P1). This suggests that relaxin may be synthesized as a large precursor, from which subsequent proteolytic cleavage might generate a family of intermediates and finally the 6,300 dalton relaxin. The three high molecular weight forms isolated from frozen pregnant sow ovaries may represent conversion intermediates of relaxin, or a family of relaxinoid hormones.

The detection of multiple high molecular weight forms of relaxin reported

here suggests that the biosynthetic pathway of relaxin may be totally different from that of insulin, despite the striking structural similarity between the two hormones. The difference may reflect their endocrine roles, besides the different embryonic origins of the pancreas and ovary. Insulin is a most important anabolic hormone, and therefore has a high synthetic turnover rate. On the other hand, relaxin appears to be accumulated continuously in the ovaries over a period of approximately 80 days of pregnancy in the pig and is then released all within a very short time just before parturition (26, 27), hence there is no need physiologically for the immediate conversion of the newly synthesized precursor as there is in the case of insulin.

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