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Naturally Occurring Porcine Relaxins and Large-Scale Preparation of the B29 Hormone†

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ABSTRACT: Porcine ovaries were collected from pregnant sows under conditions designed to keep autolysis to an absolute minimum. During the extraction the tissues were never allowed to warm up to 0 °C until submerged in 1.6 N HCl. Isolation and fractionation of the various relaxin forms became possible by application of CM-cellulose chromatography at pH 5.5 and pH 7.8, gel filtration, and high-performance liquid chromatography. The new isolation procedure has made it possible to isolate and identify Leu^{B32} relaxin. Also, [Leu-Phe^{A0}]B29 relaxin was identified and the existence of a [Leu-Phe^{A0}]B32 relaxin may be deduced from our data. Controlled digestion of B-chain-extended relaxins with carboxypeptidase A led to the large-scale production of homogeneous B29 relaxin, a suitable starting material for controlled chemical modification of porcine relaxin.

The peptide hormone relaxin causes the softening and lengthening of the symphysis pubis through significant connective tissue changes in the target region, as well as the inhibition of the uterine muscle contraction [for review, see Schwabe et al. (1978)]. Porcine relaxin (Figure 1) (Schwabe et al., 1976, 1977; Schwabe & McDonald, 1977; James et al., 1977) consists of two chains linked by disulfide bonds. The relative position of the one intrachain and the two interchain disulfide links is identical with the disulfide bond location in the pancreatic hormone insulin (Schwabe & McDonald, 1977). One significant property of porcine relaxin is the lack of homogeneity of the biologically and immunologically active material when isolated by the method of Sherwood & O'Byrne (1974), who obtained three equipotent relaxins in comparable amounts via ion-exchange chromatography. Sequence analysis of these different forms of relaxin have elucidated a constant A chain of 22 amino acids and variations in length at the C terminus of the B chain from 28 to 31 amino acid residues (Niall et al., 1980). Walsh & Niall (1980), using a small-scale extraction procedure, described B31 relaxin as the predominant form of the hormone in pig ovaries; only minimal contaminants of B29 relaxin were observed. They postulated that shortened forms are generated during the isolation procedure by proteolysis.

Using a different isolation procedure, we have recently obtained the first relaxin variant involving the A chain (Büllesbach & Schwabe, 1985). A [Phe^{A0}]relaxin was observed in considerable amounts (about 10%), and we have postulated that this [Phe^{A0}]relaxin is an intermediate in prorelaxin–relaxin conversion. We have furthermore predicted that a [Leu-Phe^{A0}]relaxin should be occurring in porcine ovaries.

Neither the variability in length of the C terminus of the B chain (Sherwood & O'Byrne, 1974; Tregear et al., 1983; Anderson, 1984) nor the extension at the N terminus of the A chain (Büllesbach & Schwabe, 1985; Schwabe, 1983) caused differences in biological activities. However, the chemical modification of relaxin and the semisynthesis of relaxin analogues would be severely hampered by the variability of available material.

The ultimate aim of the experiments presented in this paper was the production of a highly purified porcine relaxin on a preparative scale. The homogeneous relaxin could serve as defined starting material for chemical modifications for studies of the structure–function relationship of porcine relaxin and of the relaxin receptor.

Experimental Procedures

Materials

Porcine ovaries from late pregnant sows were collected in liquid nitrogen and stored at −70 °C before use. Immature ICR mice (20–25 g) were used for bioassay. Chemicals for chromatography were HPLC[1]-grade (J. T. Baker Chemicals Co., Phillipsburg, NJ) or distilled in glass quality (Burdick & Jackson, Muskegon, MI). All other chemicals were analytically pure.

Urea stock solution (7 M) was stored over a mixed-bed ion exchanger at 4 °C. Carboxypeptidase A (bovine pancreas, EC 3.4.17.1) was obtained from Schwarz Bioresearch Inc. Dialysis membrane tubing (Spectrophor 3, exclusion limit M_r 3500) was purchased from American Scientific Products (McGaw Park, IL). CM-cellulose (Whatman CM-52) was supplied by Reeve Angel (Clifton, NJ), and Sephadex products

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[1] Abbreviations: CP-A, carboxypeptidase A; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin.

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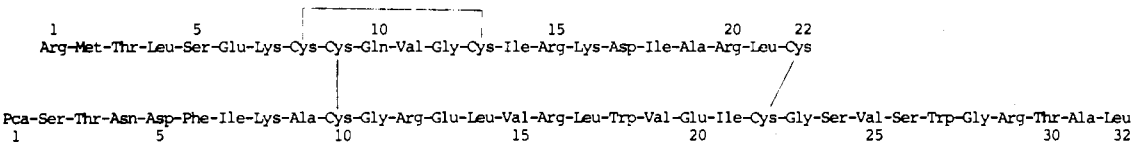

FIGURE 1: Primary structure of B32 relaxin.

were obtained from Pharmacia (Piscataway, NJ).

Methods

Cellulose–acetate electrophoresis was performed in a deluxe electrophoresis chamber (Gelman Sciences, Ann Arbor, MI); the cellulose–acetate strips (Sepraphore III, 2.5 cm × 17.1 cm) were a product of the same company. For electrophoresis at pH 4.8 (1.5 h at 200 V), we used 4 M urea and 0.1 M pyridine adjusted to pH 4.8 with acetic acid. Electropherograms at pH 8.6 were performed (3 h at 200 V) with 4 M urea, 25 mM sodium barbital, 25 mM sodium acetate, 5 g/L Na_2EDTA (disodium ethylenediaminetetraacetate), and dioxane (10 vol %).

High-Performance Liquid Chromatography (HPLC). A Synchropak RP-P column (4.1 mm × 250 mm) (Synchron Inc., Linden, IN), protected by a precolumn of Co:Pell ODS (30–38 μm) (Whatman, Clifton, NJ), was used for all relaxin separations. The column was eluted at 1.5 mL/min with two Waters HPLC pumps (Model 6000A) controlled by a Waters Model 660 solvent gradient programmer. The eluate was detected by a UV monitor at 226 nm (Uvicord S, LKB Product, Bromma, Sweden).

Amino acid analyses were performed on a Durrum D 502 analyzer (Durrum Instrument Co., Palo Alto, CA). Samples were hydrolyzed for 36 h in vacuo in 6 N HCl at 120 °C. The values for Thr and Ser are corrected.

Sequence analysis was carried out by Edman degradation in a Beckman 890 TC automatic sequencer using a 0.1 M quadrol program and polybrene to aid retention of the relaxin in the sequencer cup. PTH-amino acids were identified by HPLC according to Schwabe et al. (1984).

UV spectra were obtained on a Cary 15 recording spectrophotometer (Cary Instruments, Atlanta, GA).

Isolation of Porcine Relaxin. Frozen ovaries of pregnant sows (3 kg) were ground in a meat grinder and dropped directly into ice-cold HCl (1.6 M, 1.5 L). The mixture was further homogenized in a high-capacity Polytron (Brinkman) and kept at 4 °C for 24 h. The homogenate was mixed with acetone to a final concentration to 70%. After 3–5 h at 4 °C, the suspension was centrifuged at 3000 rpm for 15 min at 4 °C in a Beckman centrifuge (Model J-6B). The supernatant was brought up to 90% in acetone. After 60 h at 4 °C, the resulting precipitate was collected and dissolved in 500 mL of water. The pH was adjusted to 5.5 with ammonia, and the suspension was dialyzed against 3 changes of 0.025 M ammonium acetate buffer (pH 5.5) over 24 h. The suspension was centrifuged at 3000 rpm, and the supernatant was pumped directly onto a CM-cellulose column (5 cm × 16 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 5.5). Unbound protein was washed out with the same buffer. When no more UV-positive material left the column, the relaxin-containing fraction was eluted with 0.05 M ammonium acetate (pH 5.5) containing 0.2 M NaCl. Therefore, the column was washed with 0.5 M NaCl at pH 5.5. The fractions were lyophilized, dialyzed against deionized water, and lyophilized. The relaxin-containing fraction contained approximately 1 g of protein/kg of ovaries. This material (1 g) was further purified on CM-cellulose (3 cm × 25 cm), equilibrated with pH 7.8 buffer (7 M urea, 0.05 M Tris, adjusted to pH 7.8 with HCl). Relaxin was eluted with a linear gradient of NaCl (0–0.1 M NaCl, 500 mL each). Fractions of 6 mL were collected. Fractions 76–102 were pooled, acidified with acetic acid to pH 4–5, desalted on Sephadex G-25 m in 1 M acetic acid, and lyophilized (yield 250 mg/kg of ovaries).

Preparation of B29 Relaxin. The mixture of relaxins (250 mg) was dissolved in 4 mL of 0.05 M NH_4HCO_3. A freshly prepared suspension of carboxypeptidase A (2.5 mg in 1 mL of 1 M NaCl) was added, and the digest was incubated for 1 h at 37 °C. The cloudy solution was acidified with 500 μL of glacial acetic acid and applied to a Sephadex G-50 sf column (3 cm × 53 cm) equilibrated with 1 M acetic acid containing 0.15 M NaCl. Fractions 31–37 were pooled and desalted on a Sephadex G-25 m in 1 M acetic acid and lyophilized (yield about 205 mg/kg of ovaries). This material was rechromatographed on a CM-cellulose column at pH 7.8 (7 M urea, 0.05 M Tris, HCl) with a linear NaCl gradient (0–0.1 M NaCl, 500 mL each). Fractions 87–95 were pooled, acidified to pH 4–5 with acetic acid, desalted on Sephadex G-25 m in 1 M acetic acid, and lyophilized (yield 137 mg/kg of ovaries).

Radioimmunoassay of porcine relaxin was performed by using a sheep anti-porcine relaxin antibody in combination with a goat anti-γ-globulin immunosorbent cellulose. (Formyltyrosyl)relaxin (Schwabe, 1983), iodinated by the chloramine T method (Hunter & Greenwood, 1962), was used as tracer.

Mouse pubic ligament assays were performed according to Steinetz et al. (1960) with immature female ICR mice (20–25 g), which had been primed 7 days prior with 5 μg of estradiol cyclopentylpropionate in 0.1 mL of sesame oil. The doses of relaxin (0.1, 0.2, 0.4, 0.5, 0.8, and 1.2 μg) were suspended in 0.1 mL of 1% benzopurpurin 4B and injected into groups of 10–15 mice. These mice were killed 18–24 h later in an atmosphere of CO_2. The pubic joint was freed of adhering connective tissue, and the distance between the interpubic bones was measured at 10× magnification on a binocular dissecting scope fitted with an ocular micrometer and transilluminating fiber optics.

RESULTS

Porcine relaxin from pig ovaries was isolated first by acid acetone precipitation according to Doczi (1963). The acetone pellet was purified by CM-cellulose chromatography at pH 5.5 with a stepwise gradient of 0, 0.2, and 0.5 M NaCl (Schwabe et al., 1983). Relaxin was eluted with 0.2 M NaCl, and this fraction was further purified on CM-cellulose at pH 7.8 in the presence of 7 M urea, with a linear NaCl gradient (Figure 2).

All fractions were separated by size on Sephadex G-50 sf in 1 M acetic acid containing 0.15 M NaCl. The salt-free fractions of M_r 6000 were investigated in radioimmunoassay using a polyclonal antibody. Full activity was found in fractions 1, 2, 3a, and 3b, whereas all other fractions showed an RIA activity lower than 50%.

The fraction of M_r 6000 of peak 1 was of a purity better than 90% as demonstrated by cellulose–acetate electrophoresis at pH 4.8 and pH 8.6 but was not totally homogeneous in HPLC (data not shown). This fraction is probably similar to the relaxin fraction designated CM-B by Sherwood & O'Byrne (1974) and consists of relaxins shorter than B29

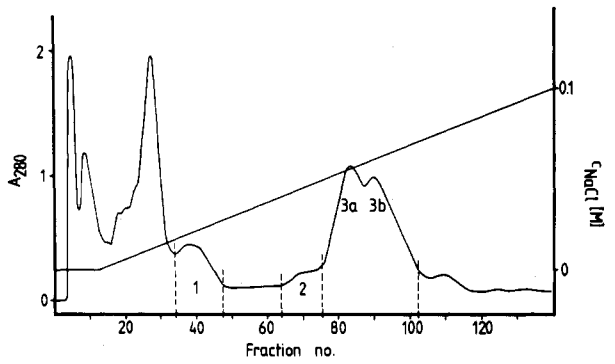

FIGURE 2: Ion-exchange chromatography of crude porcine relaxin on CM-cellulose at pH 7.8 (column, 3 × 25 cm; buffer, 7 M urea, 0.05 M Tris-HCl, pH 7.8; gradient, 0–0.1 M NaCl, 500 mL each; flow rate, 50 mL/h; fractions, 6 mL).

(Niall et al., 1980; John et al., 1981b, Schwabe, 1981) or of the desamido products of B29 and relaxins possessing C-terminal elongations (Niall et al., 1980). Of this fraction, 30 mg/kg of ovaries was obtained. The preparation was fully active in the mouse interpubic ligament assay but was not further investigated. The 6000-dalton fraction of peak 2 (Figure 2) was more than 85% enriched according to the results of electrophoresis experiments at pH 4.8 and pH 8.6. Compared with peak 1, peak 2 migrated faster to the cathode in both systems. Carboxypeptidase A digestion liberated the amino acids Leu, Ala, and Thr in the ratio 0.7:1:1 as identified by amino acid analysis. The corresponding mixture of relaxins was separated by HPLC, and one of the components was identified as [Phe^{A0}]B29 relaxin (Büllesbach & Schwabe, 1985).

The main product eluted in a double peak. Both parts of the double peak 3a and 3b (Figure 2) were better than 95% pure and were identical in electrophoretic properties at pH 4.8 and pH 8.6. Their electrophoretic mobility was also identical with that of the main product in fraction 2. After gel filtration on Sephadex G-50 sf, about 80–85% of the protein was recovered in a peak of M_r 6000. Investigations were performed with two single fractions derived from this double peak, namely, fraction 80 (from the positive slope) and fraction 95 (from the negative slope). Amino acid analysis of the gel-filtered material showed significant differences in the values of Thr, Ala, Leu, and Phe, which were distinctly higher in fraction 80 than in fraction 95. However, carboxypeptidase A liberated Thr, Ala, and Leu in the ratio 1:1:0.7 from the fraction contained in tube 80 while only 7% Thr relative to the relaxin content was liberated in fraction 95. Consequently, the main components in fraction 3a were B31 relaxin/B32 relaxin in a ratio of 3:7, and the main component in fraction 3b was B29 relaxin.

The higher Phe value in fraction 80 was likely caused by [Phe^{A0}]relaxin contamination. It seems to be very possible that [Phe^{A0}]relaxin shows the same distribution of variations at the C terminus of the B chain as does [Arg^{A1}]relaxin itself. Therefore, only [Phe^{A0}]B31 relaxin/[Phe^{A0}]B32 relaxin could be separated in peak 2 whereas the [Phe^{A0}]B29 relaxin remained as a contaminant of fraction 3a. In general, the observed double peak 3a/3b is similar to the fractionation described by Sherwood & O'Byrne (1974) using CM-cellulose chromatography at pH 5.5. From the pooled double peak, B32 relaxin could be separated via HPLC on reversed-phase columns (Figure 3A), and it eluted in the same position as [Phe^{A0}]B29 relaxin. B29, B30, and B31 relaxins could not be separated under these conditions. Since the ratio of the peaks is 65:35 before carboxypeptidase A digestion (Figure 3A) and 93:7 after carboxypeptidase A digestion (Figure 3B), the amount of B32 relaxin is estimated to be about 28% of the double peak.

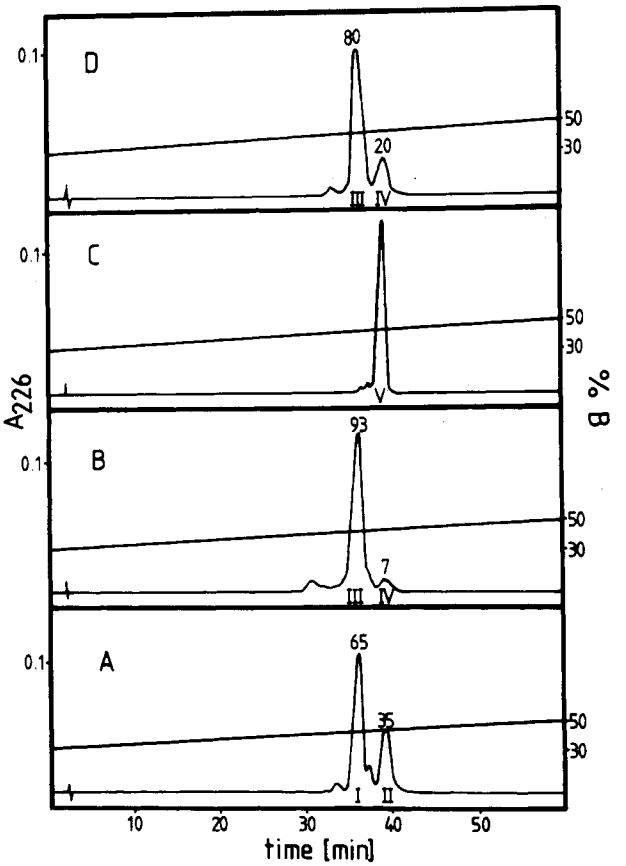

FIGURE 3: HPLC of (A) pool 3a/3b of Figure 2, (B) carboxypeptidase A digestion of (A), (C) B32 relaxin after isolation by preparative HPLC, and (D) carboxypeptidase A digestion of (C) (column; Synchropak RP-P; solvent system A; 0.1% trifluoroacetic acid in water; solvent system B, 0.1% trifluoroacetic acid in 80% acetonitrile; linear gradient of 30–50% B in 1 h; flow rate, 1.5 mL/min).

Table I: Amino Acid Analyses of Porcine Relaxin

	B32 relaxin		B29 relaxin,	[Leu-Phe^{A0}]B29 relaxin,
	6 N HCl	CP-A[a]	6 N HCl	6 N HCl
Asp	2.97 (3)		3.19 (3)	3.25 (3)
Thr	2.58 (3)	0.88 (1)	2.36 (2)	2.11 (2)
Ser	4.21 (4)		3.36 (4)	3.44 (4)
Glu	5.13 (5)		5.66 (5)	5.79 (5)
Pro			0.15 (0)	
Gly	4.02 (4)		4.15 (4)	3.99 (4)
Ala	3.33 (3)	1.00 (1)	1.64 (2)	2.02 (2)
Cys	5.67 (6)		6.49 (6)	5.84 (6)
Val	4.18 (4)		4.00 (4)	4.06 (4)
Met	0.64 (1)		0.91 (1)	0.93 (1)
Ile	3.85 (4)		3.86 (4)	4.08 (4)
Leu	5.08 (5)	0.84 (1)	4.23 (4)	4.91 (5)
Phe	0.97 (1)		0.95 (1)	1.95 (2)
Lys	3.00 (3)		3.15 (3)	3.20 (3)
Arg	5.25 (5)		6.16 (6)	6.02 (6)

[a] CP-A, carboxypeptidase A digestion.

We have isolated B32 relaxin by preparative HPLC. This preparation was found to be about 95% pure after electrophoresis in two different systems and after analytical HPLC (Figure 3C). The amino acid composition is shown in Table I. Carboxypeptidase A liberated Leu, Ala, and Thr in almost equimolar amounts (see Table I). However, the separation of the resulting mixture by HPLC showed that only 80% of the relaxin migrated in the position of B29 relaxin, whereas

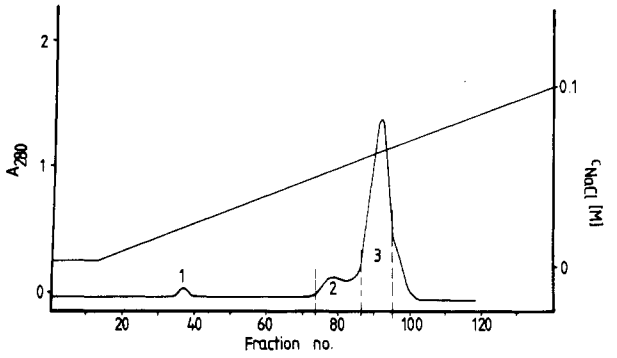

FIGURE 4: Ion-exchange chromatography of crude B29 relaxin on CM-cellulose at pH 7.8 (column, 3 × 25 cm; buffer, 7 M urea, 0.05 M Tris-HCl, pH 7.8; gradient, 0–0.1 M NaCl, 500 mL each; flow rate, 50 mL/h; fractions, 6 mL).

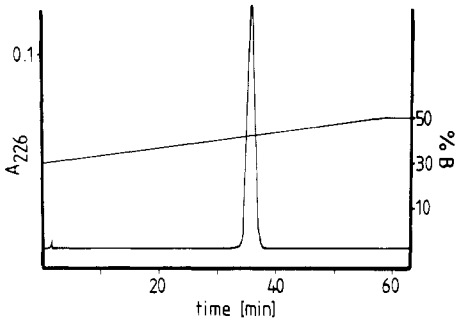

FIGURE 5: HPLC of purified B29 relaxin (column; Synchropak RP-P; solvent system A, 0.1% trifluoroacetic acid in water; solvent system B, 0.1% trifluoroacetic acid in 80% acetonitrile; linear gradient, 30–50% B in 1 h; flow rate, 1.5 mL/min).

20% remained in the position of B32 relaxin (Figure 3D). The latter was identified as [Phe^{A0}]relaxin by its elution profile in HPLC.

Preparation and Characterization of B29 Relaxin. Preparative carboxypeptidase A digestion increased the amount of B29 relaxin available for further studies by a factor of 2. Shortening of the molecule in the C-terminal region of the B chain does not affect the bioactivity of the hormone (Sherwood & O'Byrne, 1974; Tregear et al., 1983; Anderson, 1984). The digest was performed with the pooled and salt-free double peak 3a/3b (Figure 2), in 0.04 M NH_4HCO_3/0.2 M NaCl for 1 h at 37 °C and with an enzyme to substrate ratio of 1:100. The resulting B29 relaxin, separated by gel filtration on Sephadex G-50 sf in 1 M acetic acid containing 0.15 M NaCl, was finally purified by rechromatography on CM-cellulose at pH 7.8 (Figure 4). Electrophoretical experiments suggested that a des-Arg^{B29}-relaxin in peak 1 (~1%) (Figure 4) was caused by a carboxypeptidase B contaminant in carboxypeptidase A preparations. Peak 2 eluted in the same position as B32 relaxin/B31 relaxin. However, these relaxin molecules cannot be present because a carboxypeptidase A digestion of this material did not cause liberation of amino acids. The main component of peak 2 was identified as [Phe^{A0}]B29 relaxin, which was contaminated with B29 relaxin and some other impurities (Büllesbach & Schwabe, 1985). The resulting main peak (Figure 4) consisted of B29 relaxin, which was obtained in a yield of 130–140 mg/kg of ovaries. This form of relaxin (B29) was characterized by cellulose–acetate electrophoresis at pH 4.8 and pH 8.6. A small impurity of less than 5% was observed in the electrophoresis at pH 4.8. Amino acid analysis yielded the expected amino acid composition (Table I). No tyrosine or histidine was present, but the proline content was less than 0.2 residue instead of zero. During HPLC, the B29

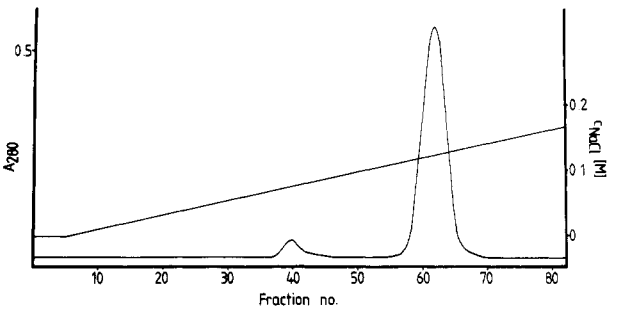

FIGURE 6: CM-cellulose chromatography of 20 mg of purified B29 relaxin at pH 5.0 (column, 3 × 25 cm; buffer, 7 M urea, 0.05 M CH_3COONH_4–CH_3COOH, pH 5.0; gradient, 0–0.2 M NaCl, 500 mL each; flow rate, 50 mL/h; fractions, 6 mL).

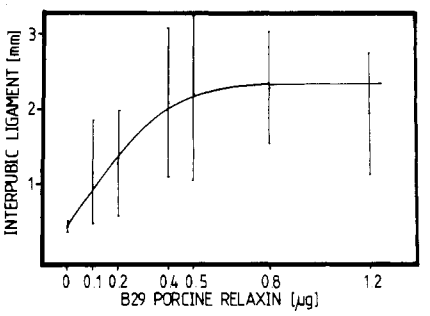

FIGURE 7: Mouse interpubic ligament assay of purified B29 relaxin. Each experimental point represents 15 animals.

relaxin fraction showed a single peak (Figure 5). The molecular weight of B29 relaxin was determined by fast atom bombardment (FAB) mass spectrometry to be 5814.8 for the protonated molecule ion, which is identical with the theoretically calculated value (Fenselau, 1984).

The impurity observed in electrophoresis at pH 4.8 can be removed by rechromatography on CM-cellulose at pH 5.0 (Figure 6), which reduces the proline content to zero (after further HPLC separation). Other minor impurities (approximately 1%) can be caused by carbamylation of an amino group during the isolation procedure.

UV spectroscopy of a 0.1% solution of B29 relaxin (dried over phosphorus pentoxide) in water resulted in a specific extinction coefficient of $A_{282}^{0.1\%} = 1.62$ cm^2 mg^{-1} (theoretical 1.92 cm^2 mg^{-1}) with a maximum to minimum ratio (A_{282}:A_{250}) of 2.08.

B29 relaxin was tested in the mouse interpubic ligament assay (Steinetz et al., 1960). The dose–response curve of the widening of the symphysis pubis is shown in Figure 7. A half-maximum response was obtained with 0.25 µg/mouse. B29 relaxin was fully competent in the radioimmunoassay using polyclonal sheep anti-relaxin antibodies.

Isolation and Characterization of [Leu-Phe^{A0}]B29 Relaxin. All fractions containing [Phe^{A0}]relaxin (fraction 2 in Figure 2 and fraction 2 in Figure 4) were digested with carboxypeptidase A, separated by gel filtration on Sephadex G50 sf in 1 M acetic acid/0.15 M NaCl, and followed by HPLC on reversed-phase columns as described by Büllesbach & Schwabe (1985). The most hydrophobic main peak (Figure 8A) was identified as [Phe^{A0}]B29 relaxin. However, this peak eluted with a minor shoulder which was cut off during the fractionation of [Phe^{A0}]B29 relaxin. The rechromatography of this shoulder resulted in a double peak. The most hydrophobic peak (Figure 8B) was isolated in a yield of 0.1 mg/kg of ovaries and was characterized by electrophoresis at pH 4.8 and pH 8.6, by amino acid analysis after acid hydrolysis, and by sequencing. Compared to B29 relaxin, no difference in

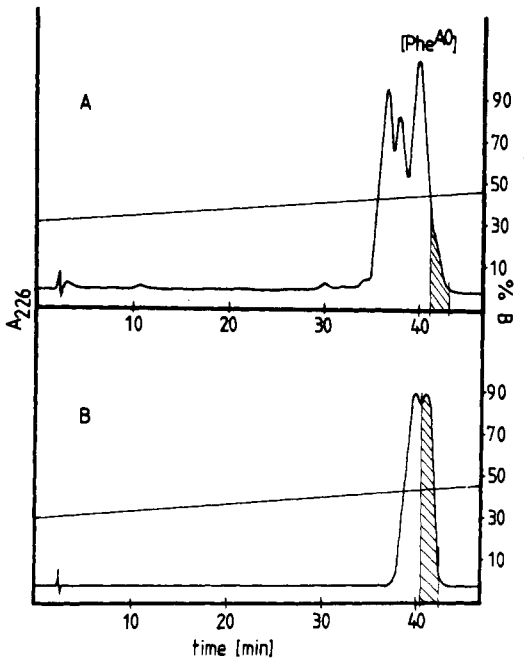

FIGURE 8: (A) HPLC of pooled [Phe^{A0}]B29 relaxin containing fractions and (B) rechromatography of the trailing portion of the [Phe^{A0}]relaxin peak (column, Synchropak RP-P; solvent system A, 0.1% trifluoroacetic acid in water; solvent system B, 0.1% trifluoroacetic acid in 80% acetonitrile; linear gradient; 30–50% B in 1 h; flow rate, 1.5 mL/min).

electrophoretic mobility was observed, and the amino acid analysis (Table I) resulted in the amino acid composition of B29 relaxin with increased values of Phe and Leu. Automatic Edman degradation was performed with the intact molecule. Since the B chain of the native porcine relaxin is blocked via pyroglutamine, only the sequence of the A chain was obtained. However, this A chain showed an extension of Leu-Phe at its N terminus whereas all other amino acids (the cystines were not identified) were identical with the described sequence of the A chain (Schwabe et al., 1976; James et al., 1977).

Discussion

The in vivo conversion of prorelaxin to relaxin appears to be much less stringently controlled than is the conversion of other prohormones to the active hormones (Steiner et al., 1980), hence the appearance of relaxins of different lengths either at the N-terminal end of the A chain, the C terminus of the B chain, or both. Some of these relaxin forms occur in significant amounts. They could well have escaped detection if the amount of starting material used was too low to detect a fraction as small as 10% of the main product or if a purification method was employed by which the fraction could not be separated. Thus, Walsh & Niall (1980) identified B31 relaxin as the major product, but did not detect significant amounts of B32 relaxin or any of either [Leu-Phe^{A0}]relaxin or [Phe^{A0}]relaxin. In contrast, we observed equal amounts of B29 and a mixture of B31 relaxin/B32 relaxin. Peculiarly, B30 relaxin is barely observed. Relaxins possessing fewer than 29 amino acids in the B chain and desamido products of B29 to B32 relaxins were observed in distinctly lower amounts than reported by Sherwood & O'Byrne (1974).

The N-terminal variation of the B chain of relaxin may be indicative of the activation process that leads to the removal of the rather large C peptide. The nucleotide sequence (Haley et al., 1982) suggests that four basic residues preceding Leu-Phe direct the action of a trypsin-like endopeptidase. The first product is [Leu-Phe^{A0}]relaxin, which contains 24 amino acids in the A chain as do all other known relaxins (John et al., 1981; Gowan et al., 1981). This molecule is then shortened by an intracellular aminopeptidase, one residue at a time, to yield [Arg^{A1}]relaxin.

In the absence of the expected pair of basic amino acids in the cleavage region of the B chain–C peptide junction, enzymatic hydrolysis of the Leu^{B32}/Ser^{B33} bond was proposed by Haley et al. (1982). First evidence for the occurrence of B32 relaxin is described by Niall et al. (1980). However, the isolation of the B32 relaxin in a state of high purity suggests a mechanism of cleavage involving Leu^{B32}. Our experiments suggest that Arg^{B29} may be a natural stopping point for carboxypeptidase action in that region of the prohormone. The occurrence of B32, B31, and B29 relaxins certainly suggests such a mechanism while the virtual absence of B30 relaxin makes it seem possible that some conversion could occur directly by a trypsin-like endopeptidase attack at Arg^{B29}.

The isolation procedure of porcine relaxin using CM-cellulose at pH 7.8 allowed the separation (from other relaxin) of [Phe^{A0}]relaxin, which eluted in two different peaks (peaks 2 and 3a in Figure 2). It seems that this separation was possible because of the variations of the C-terminal ends of the B chain. Thus, the isolation of [Phe^{A0}]B29 relaxin by HPLC became possible after the C-terminal variations had been removed by carboxypeptidase A treatment (Büllesbach & Schwabe, 1985). It is remarkable that [Leu-Phe^{A0}]relaxin eluted in a shoulder of the [Phe^{A0}]relaxin peak. Since carboxypeptidase A treatment liberated Leu, Ala, Thr from the phenylalanine-enriched relaxin material, it is very possible that [Phe^{A0}]relaxin and [Leu-Phe^{A0}]relaxin existed as their B32 derivatives, the largest relaxin molecules in porcine relaxin preparations.

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Preparation and Properties of α- and ϵ-Amino-Protected Porcine Relaxin Derivatives†

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ABSTRACT: The chemical modification of the amino groups of B29 porcine relaxin resulted in pure derivatives of $N^{\alpha A1}$-citraconyl-B29 relaxin, $N^{\epsilon A7},N^{\epsilon A16},N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 relaxin (Msc_3-relaxin), and $N^{\alpha A1},N^{\epsilon A7},N^{\epsilon A16},N^{\epsilon B8}$-tetrakis[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 relaxin (Msc_4-relaxin). $N^{\alpha A1}$-Citraconyl-B29 relaxin was obtained after selective deprotection of fully acylated B29 relaxin derivatives. The quantitative reaction of $N^{\alpha A1}$-citraconylrelaxin with [[(methylsulfonyl)ethyl]-oxy]carbonyl succinimide ester followed by deprotection of the citraconyl group resulted in $N^{\epsilon A7},N^{\epsilon A16},N^{\epsilon B8}$-$Msc_3$-B29 relaxin, the starting material for selective chemical modifications at the N terminus of the relaxin A chain. In mouse interpubic ligament assay both Msc_3 and Msc_4 derivatives of relaxin showed a bioactivity of 30%, while in the case of $N^{\alpha A1}$-citraconyl-B29 relaxin the bioactivity was reduced to 15%. When compared with unmodified relaxin, only the circular dichroic spectrum of $N^{\alpha A1}$-citraconyl-B29 relaxin revealed significant differences. Therefore, the loss in bioactivity of the $N^{\alpha A1}$-citraconyl-B29 relaxin seems to be related to the structural changes caused by the introduction of a negative charge at the N terminus of the A chain.

Relaxin, a hormone of pregnancy, consists of two peptide chains held together by disulfide links. Porcine relaxin is variable in the region of the prohormone–hormone junctions at the N terminus of the A chain (Büllesbach & Schwabe, 1985a,b) and at the C terminus of the B chain (Niall et al., 1980; Walsh & Niall, 1980; Büllesbach & Schwabe, 1985b). Since these variations cause no differences in biological activity (Sherwood & O'Byrne, 1974; Tregear et al., 1983; Anderson, 1984; Büllesbach & Schwabe, 1985a), it is reasonable to assume that these regions do not participate in receptor binding.

It is not clear which part of the molecule takes part in hormone–receptor interaction, because (i) no crystal structure could be obtained until now, (ii) relaxin structures of only five different species are known (Schwabe et al., 1976, 1977; James et al., 1977; Gowan et al., 1981; Hudson et al., 1981, 1983, 1984; E. E. Büllesbach et al., unpublished results), and (iii) chemical modification is hampered by the variability of the C-terminal portion of porcine relaxin.

On the basis of the similarity of the disulfide links and other structural features between relaxin and insulin (Schwabe & McDonald, 1977), an insulin-like structure has been predicted for relaxin. Model-building studies (Bedarkar et al., 1977) and computer-graphic studies (Isaacs et al., 1978) have shown that porcine relaxin fits, without strain, into the three-dimensional coordinate system of porcine insulin. Circular dichroism studies of relaxin and insulin also showed a high similarity between both hormones (Schwabe & Harmon, 1978; Rawitch et al., 1980; Du et al., 1982). However, the surface of a relaxin molecule is quite different from the surface of an insulin monomer, and therefore, no biological and immunological cross-reactivities are observed (Rawitch et al., 1980).

On the basis of the insulin-like structure and the primary structure of relaxins of different species, the conserved residues on the surface of the molecule seem to be hydrophilic and basic. Candidates for receptor binding (Figure 1) were described in different papers (Gowan et al., 1981; Bedarkar et

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