Purification and Characterization of Pituitary Bovine Somatotropin*

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Bovine somatotropin (bST) has been isolated from pituitary glands and compared in a variety of chemical analyses and bioassays with somatotropin derived from recombinant Escherichia coli. Comparison of pituitary extracts and purified bST by Western blot analysis of two-dimensional gels suggested that the immunoreactive somatotropin species present in the extract were also present in the purified material, with no significant losses or degradation as a result of the purification method. NH2-terminal sequence analysis indicated the presence of equal quantities of Ala-Phe-Pro-Ala-Met-Ser-Leu-Ser- and Phe-Pro-Ala-Met-Ser-Leu-Ser- sequences. The Met-Ser-Leu-Ser-NH2-terminal sequence, a degradation product observed in NIH standard lots, was not detected. Assays of bioactivity in a bovine liver receptor-binding assay and in a female rat growth assay showed pituitary bST and recombinant methionyl-bST to be equipotent. Tryptic maps and sequence analysis of pituitary-derived somatotropin suggest the presence of isoaspartate derivatization at Asp.

Bovine somatotropin has been isolated from pituitary glands by a variety of methods, resulting in preparations with varying degrees of heterogeneity (1). Deletion of NH2-terminal residues has been observed in somatotropin prepared from acidic extracts and appears to be the result of proteolysis during isolation (2). However, the amino-terminal sequence of highly purified pituitary bovine somatotropin has been shown to contain only Ala-Phe-Pro-Ala-Met-Ser-Leu-Ser- and Phe-Pro-Ala-Met-Ser-Leu-Ser- in approximately equimolar amounts, which appear to result from signal peptide processing during secretion (3). Other observed heterogeneity includes an allelic variation at position 126, which leads to a 2:1 ratio of leucine to valine in preparations from pooled glands (4). In addition, bovine somatotropin fractions from anion-exchange chromatography have been shown to have different biological activities in a panel of bioassays (5). Thus, bovine somatotropin appears as a mixture of related polypeptides that may differ in function or in site of action.

To characterize pituitary somatotropin better, we describe here a purification method that allows isolation of highly purified protein in gram quantities. The methodology employed resulted in a 2-4-fold greater yield of somatotropin than reported previously (1), while maintaining excellent purity. Extensive biochemical analysis of the pituitary-derived material has been carried out to identify microheterogeneity undetected previously. In order to assess the effects of such microheterogeneity on activity, bovine somatotropin from recombinant Escherichia coli has been compared with pituitary somatotropin in several biological assays.

EXPERIMENTAL PROCEDURES

Pituitary bST Purification—Bovine pituitaries were obtained within 1 h of slaughter, placed on ice for 2-4 h, then stored frozen at -80°C. All subsequent purification steps were carried out at 0-4°C. The initial extraction of 33 whole pituitary glands (66 g, wet weight) in 150 ml of extraction buffer was at low speed for 10 min using a Waring blender controlled by a rheostat. Extraction buffer was 4.5 M urea, 50 mM Tris, pH 8.8, 1 mM phenylmethylsulfonyl fluoride 1 mM benzamidine HCl, and 4 mM EDTA. The resulting suspension was further homogenized for 10 min using an Ultra-turrax (Tekmar Co.). This extract was subjected to centrifugation for 15 min at 9,000 rpm in a Sorvall GS-3 rotor. The resulting clear red supernatant was removed by pipette from beneath a white floating layer of lipid-like material. The white layer was discarded, the pellet was extracted as before for 10 min using the Ultra-turrax, supernatant was collected as above, and pooled with the first supernatant.

This extract was adjusted to a pH of 10.2, deionized, and subjected to centrifugation for 20 min in a Beckman Ti-45 rotor at 25,000 rpm to remove turbidity. The solution was loaded onto a 10-cm diameter × 12-cm DEAE-cellulose column (Whatman DE52) which had been equilibrated in 4.5 M urea, 50 mM Tris base. Elution was carried out with a 14-liter gradient composed of equal volumes of equilibration buffer and 4.5 M urea, 50 mM Tris-HCl, pH 8.85, at a flow rate of 0.4 liters/h. Fractions were assayed by reversed-phase HPLC and SDS-PAGE, and those fractions containing bST were pooled and then concentrated using an Amicon YM-10 membrane in a stirred cell apparatus.

Buffer exchange of the pool was performed using a Sephadex G-25 column equilibrated and eluted in 4.5 M urea, 5 mM sodium phosphate, pH 6.9. The G-25 pool was loaded onto a 5-cm diameter × 12-cm carboxymethyl (CM)-cellulose column equilibrated in the 4.25-cm column buffer and eluted with a 2-liter gradient from 5-25 mM sodium phosphate at 0.3 liter/h. Fractions containing bST were pooled and dialyzed versus 5 mM sodium bicarbonate, pH 10, and finally dialyzed versus water to reduce the sodium bicarbonate concentration to <1% of protein mass. The dialysate was lyophilized and stored at -20°C.

Reversed-phase HPLC—Reversed-phase HPLC analysis of somatotropin was performed using a 0.4 × 10-cm Econosphere 300 C8 cartridge column with a 0.4 × 1.0-cm C4 guard cartridge (Alttech), using an acetonitrile gradient in 0.1% trifluoroacetic acid. Elution was monitored by UV absorbance at 220 nm. Quantitation of bST samples was by comparison of the bST peak area with a standard curve prepared using methionyl-bST.

SDS-PAGE—Gel electrophoretic analysis for routine screening of column fractions was performed using the Pharmacia LKB Biotech.

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† The abbreviations used are: bST, bovine somatotropin; HPLC, high pressure liquid chromatography; RP, reversed-phase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.
nology Inc. Phastsystem™ with 10–15% acrylamide SDS gels and silver staining. Electrophoresis and staining conditions were as described in the instrument manual. Gel analysis of samples for Western blotting and to determine degree of purity was on 15% total acrylamide, 2.7% bisacrylamide gels as described by Laemmli (6).

IEF Analysis—Isoelectric focusing analysis was performed by a standard agarose gel IEF method (7) employing pH 5–9 ampholytes.

Two-dimensional PAGE—First-dimension analyses using isoelectric focusing (IEF) were carried out in polyacrylamide (4% total acrylamide, 5.4% bisacrylamide) containing 9.1 M urea, 0.5% (w/v) Nonidet P-40, and 2.1% (w/v) ampholytes (LKB, pH 3.5–9.5) for 8000 V-h. Second-dimension analyses were performed using SDS-PAGE in gels containing 12.5% total acrylamide, 2.67% bisacrylamide. Samples were applied to the IEF gels as a solution containing 9.5 M urea, 5% (w/v) 2-mercaptoethanol, and 1% (w/v) ampholytes (LKB, pH 3.5–9.5). Western blot analyses of the two-dimensional gels were performed using a modification2 of the procedure described by Reinhard and Malamud (8).

NH2-terminal Sequence Determination—Automated Edman degradation was used to determine the NH2-terminal protein sequence. An Applied Biosystems model 470A gas-phase Sequencer was employed (9). The respective phenylthiohydantoin derivatives were identified by on-line RP-HPLC employing an Applied Biosystems model 120A PTH Analyzer fitted with a Brownlee 2.1-mm inner diameter PTH-C18 column.

Total Amino Acid Compositional Analysis—Compositional amino acid data were collected from samples that had been subjected to hydrolysis (6 M HCl, evacuated sealed tubes, 24 h, 110 °C). All analyses were performed with ninhydrin in a Beckman model 6300 Auto-analyzer.

Tryptic Mapping—Trypsin digestion was performed on purified protein without reduction and alkylation, and the resulting peptides were separated by reversed-phase HPLC (10).

Spectral Characterization—Lyophilized protein was dissolved in water and filtered through a Millex-GV 0.22-μm filter unit. Absorbance spectra were measured with a Perkin-Elmer λ-3840 diode array spectrophotometer. Fluorescence spectra were measured with an SLM 4800 spectrofluorometer. All spectra were obtained within 1 h after filtration.

Receptor-binding Assay—Biological activity of bovine somatotropin was measured by a homologous somatotropin radioreceptor assay utilizing recombinant bST essentially as developed by Haro et al. (11). A procedure reported by Tsushima and Friesen (12) was modified to prepare microsomal fractions of bovine liver. Radioreceptor assay tubes (12 × 75-mm polypropylene) contained 350 μl of assay buffer (25 mM Tris-HCl, 10 mM MgCl2, 0.1% bovine serum albumin, pH 7.6), 100 μl of standard hormone or unknown, 100 μl of assay buffer containing 90000 cpm of radiiodinated recombinant somatotrophin (approximately 80 μCi of 125I/g), and 150 μl of bovine liver microsomal preparation. Recombinant bovine somatotropin was diluted in assay buffer to concentrations ranging from 0.15 to 40,000 ng/ml for use as the radioreceptor assay standard. The recombinant somatotropin was radiiodinated using lactoperoxidase Radioreceptor assay tubes were incubated at room temperature overnight on a platform orbital shaker. Incubation was stopped by the addition of 3 ml of ice-cold assay buffer. Bound 125I-recombinant somatotropin was separated from unbound by centrifugation for 45 min at 2000 × g. Supernatant was decanted, and the pellet was counted in a γ-counter.

Solid-state NMR—Cross-polarization magic angle spinning 31P NMR spectra were collected on a homemade solid-state NMR spectrometer operating at a proton resonance frequency of 127 MHz. All spectra were collected following 2-ms matched 1H-31P spin-lock contacts with high power (60 kHz) proton dipolar decoupling. Samples were spun at the magic angle (54.7°) with respect to the static magnetic field at speeds of 1–3 kHz in a double-bearing rotor system.

RESULTS

Purification of Pituitary bST—The purification scheme for bovine somatotropin is summarized in Table I. The overall yield was 80%, which extrapolates to 7.8 mg of recoverable somatotropin per g (wet weight) of tissue. In trial pituitary extractions, the yield of total protein and of somatotropin was greater by 2-fold in the presence of urea than with 50 mM Tris-HCl, pH 8.0, alone.

2 F. Rowold, personal communication.

### Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume [bST]</th>
<th>Total bST</th>
<th>% Recovery</th>
</tr>
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<tr>
<td>Tissue extract</td>
<td>585</td>
<td>mg/ml</td>
<td>mg</td>
</tr>
<tr>
<td>Mixed bed ion-exchange</td>
<td>1900</td>
<td>0.34</td>
<td>646</td>
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<td>616</td>
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<td>Sephadex G-25</td>
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<td>0.63</td>
<td>599</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>440</td>
<td>1.17</td>
<td>515</td>
</tr>
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</table>

FIG. 1. Reversed-phase HPLC analysis of pituitary bST. A 50-μl sample of the CM-cellulose chromatography pool (1.0 mg/ml [bST]) was injected onto a C8 column (Econsphere 300 C8, 4.5 × 100 mm, with a 10-mm C4 guard cartridge, Alltech). Gradient elution was accomplished with an acetonitrile gradient in 0.1% trifluoroacetic acid. Retention times are printed immediately after each peak. Peaks 1–4 were collected, dried under vacuum in a Speed-Vac (Savant), and analyzed by SDS-PAGE and Western blotting.

Somatotropin was eluted from a DEAE-cellulose column between 150 and 800 micro-Siemens/cm in conductivity. Under these chromatography conditions, the majority of soluble protein was retained on the column. Analysis of peak fractions by SDS-PAGE revealed the presence of several contaminating bands (data not shown). Therefore, an additional chromatography step was required.

The CM-cellulose conditions of Spitsberg (13) were adapted for use in 4.5 M urea and resulted in significant further purification of bST. Fractions across the peak of UV absorbance were assayed by SDS-PAGE (reducing) and were found by silver staining to contain a single band of Mr, 22000 (data not shown).

CM-cellulose fractions from 42 to 59 were pooled, dialyzed as described under "Experimental Procedures," and lyophilized. Reversed-phase HPLC analysis of the CM-cellulose pool resulted in the chromatogram in Fig. 1. Peaks 1–4 were present under the indicated gradient conditions of 23.7, 25.5, 26.4, and 27.6-min retention times, respectively. The peaks were present in the same ratios throughout purification, regardless of column loading, suggesting that these peaks were not artifacts of the chromatography conditions but represented related somatotropin forms.

Fig. 2a contains SDS-PAGE data for the CM-cellulose pool and for the individual RP-HPLC peaks 1, 2, 3, and 4 from Fig. 1. Only one major band was visible by silver staining, at Mr, 22000 in the reducing lanes of the gel. Note that bST in the nonreducing lanes migrated at an abnormally low Mr, suggesting that disulfide bonds hold the protein in a compact structure. Fig. 2b contains a Western blot of the gel probed with antibody raised against recombinant bST. A single immunoreactive band is present in the reduced CM-cellulose pool material. The Western blot also indicates the presence of a small percentage of immunoreactive protein of dimer molecular weight in the nonreducing lanes of the CM-cellulose pool. In Fig. 2b, lanes 5–7 (reducing) and 13-15 (nonreducing) correspond to the RP-HPLC peaks 2, 3, and 4 in Fig. 1. The immunoblot data suggested that the HPLC peaks were pre-
dominantly bST and also that trace amounts of immunoreactive bST species of apparent M, greater and less than 22,000 were generated by the RP-HPLC isolation conditions.

Chemical Analysis of Pituitary bST—Analysis by IEF gels was carried out as an assay for charge heterogeneity. Fig. 3 shows the IEF pattern of pituitary somatotropin at various stages of purification and also compares pituitary bST, recombinant methionyl-bovine somatotropin, and NIH standard bST received from A. Parlow (Harbor-UCLA Medical Center). In agreement with previous reports (1, 13), pituitary bST prepared by the method described in this report contained only two IEF bands, which correspond to the alanine (pI = 8.0) and phenylalanine (pI = 7.8) NH2-terminal species described below. Purified recombinant somatotropin contained minor amounts of at least three isoforms, whereas the NIH standard bST contained numerous bands in addition to the two bands corresponding to bST.

To ascertain whether our preparation of bST contained all of the immunoreactive bST components present in the original pituitary extract, two-dimensional gel electrophoretic analysis was performed as described under “Experimental Procedures.” The electrophoretic pattern for the pituitary extract was compared with the purified product by silver staining and by Western blot analysis using an anti-bST antibody probe (Fig. 4, a–d). The silver-stained gels were used to compare the relative abundance of somatotropin species, and Western blot analysis was used to identify qualitatively immunoreactive bST species present.

Comparison of Fig. 4, a–d, has clearly identified bST as a basic protein of M, 22,000 which is abundant in the pituitary extract (marked by an arrow). Direct comparison of the pituitary extract and purified bST gels indicated that no significant loss of immunoreactive bST occurred during purification. The Western blot data in these gels show two or three acidic immunoreactive bST species in the extract which are not present in the purified material (Fig. 4, c and d). However, the relative amount of these components is less than 5% of the total mass of bST, as judged by silver-staining intensity. Therefore, the goal of obtaining a purified bST sample that was representative of the material present in the pituitary extract has been attained.

Two-dimensional gel analysis was also performed on the NIH standard pituitary bST (from A. Parlow) as shown in Fig. 4, e–f. The Western blot of this gel (Fig. 4f) displayed trace amounts of several immunoreactive species of M, lower than 22,000, indicating the presence of proteolytic cleavages in the protein. The silver-stained gel, however, indicated that the des(1–4) bST proteolysis product was the major contaminant by mass, with the other species present only in trace amounts. From the relative intensities of the two spots in Fig. 4e, the des(1–4) bST (lower band) was equimolar with full length bST, a result also supported by NH2-terminal sequencing of the mixture (Table II).

NH2-terminal amino acid sequence of the pituitary bST CM-cellulose pool material revealed two sequences: Ala-Phe-Pro-Ala-Met-Leu-Ser-Leu-Ser- (36%) and Phe-Pro-Ala-Met-Ser-Leu-Ser- (64%). This heterogeneity at the NH2 terminus of bST has been shown to result from proteolysis at successive peptide bonds during removal of the signal peptide of the somatotropin precursor protein during secretion (5). No other sequences were detectable. In particular, the Met-Ser-Leu-Ser- and Ser-Leu-Ser-Gly-Leu- species detected in pituitary bST samples purified by other methods (14) were not present in the pituitary bST preparation of this study.

Tryptic mapping by reversed-phase HPLC has proven to be an analytical tool with high resolution for amino acid sequence heterogeneity in proteins (15). Pituitary somatotropin was digested with trypsin, and the resulting digest was analyzed by RP-HPLC. Individual peaks were isolated and identified by amino acid composition or by NH2-terminal sequencing, as necessary. Fig. 5 contains the tryptic map of bST as well as a map of the Ala1-Val28 variant of somato-
tropin, which was produced by recombinant DNA technology.

Two peaks are indicated by arrows in Fig. 5 which were present in pituitary bST but not in the recombinant material. The peaks were isolated and subjected to amino acid composition and sequence analysis. Compositional analysis identified the peaks as position 125-132 fragments Glu-Val-Glu-Asp-Gly-Thr-Pro-Arg and Glu-Leu-Glu-Asp-Gly-Thr-Pro-Ala, which contain the allelic Leu-Val variation at position 126. However, when these fragments were sequenced, the observed signal fell to zero at the 128 position, which would not occur for fragments containing a normal asparagine residue. This behavior in Edman degradation analysis is consistent with the presence of a "2-linked" isoaspartic acid at position 128, as discussed below. From the relative peak areas in the tryptic map, approximately 20% of protein molecules contained the putative isoaspartate at position 128. Note that the tryptic map was generated from the mixture of bST isoforms present in the CM-cellulose pool. Work is in progress to determine whether one of the RP-HPLC peaks shown in Fig. 1 is enriched in the isoaspartate 128 bST species.

As shown by Geiger and Clarke for model peptides (16), isoaspartyl formation from asparagine residues does not appear to result in a change in electrophoretic mobility. In Fig. 3, only two bands were observed, and these bands correspond to somatotropin having alanine and phenylalanine NH2 termini (17). The IEF gel was run at sufficiently high loading to detect an isoaspartyl derivative that was 20% of the protein mass, but no additional bands were observed. Thus far, only analytical reversed-phase HPLC of tryptic peptides has successfully resolved bST and the putative isoaspartyl derivative at Asp128.

Analyses of absorbance and fluorescence properties of pituitary bST and recombinant methionyl-bST were performed, and small but distinct differences in spectral properties were found. The A280/A390 ratios were distinctly different, with values of 1.456 and 1.556 for the pituitary and recombinant proteins, respectively (data not shown). Fluorescence spectra for the two proteins showed that the pituitary-derived protein has a higher relative quantum yield and possibly a higher emission maximum than recombinant somatotropin. Changes in both absorbance and fluorescence spectra with time in dilute aqueous solutions showed a greater tendency for the methionyl-bST to aggregate, possibly due to the presence of NH2-terminal methionine or to the relatively greater amount of isoelectric variants in the recombinant protein, as shown in Fig. 3. The possible role of isoaspartate at position 128 or...
of the position 126 allelic variation upon the spectral properties of the protein has not yet been determined.

Due to the extensive homology between prolactin and somatotropin (18) and the possibility of copurification of the two proteins, it was desirable to perform an independent assay of the purified pituitary bST for contaminating prolactin. Reversed-phase HPLC analysis was performed, and no peak was detected in the bST chromatogram at the retention time expected for prolactin (chromatogram not shown), even at column loadings that would detect prolactin at the 1% level. In a Western blot assay of pituitary bST for prolactin using an antibody raised against highly purified prolactin, a small but distinct signal was observed for prolactin. However, the presence of trace amounts of somatotropin in the prolactin used for antibody production cannot be ruled out. The possibility of cross-reactivity of the anti-prolactin antibody for bST in this assay also exists. Collectively, the HPLC and Western blot assays have shown the pituitary bST preparation to be essentially free of prolactin.

Phosphorylation in vivo has been postulated as a mechanism for attenuation of activity of somatotropin and other pituitary hormones (19). Therefore, analysis of phosphorylation in pituitary bST has been performed using solid-state 31P NMR. In contrast to a previous report of the presence of phosphate esters in ovine somatotropin (20) from an NIH standard lot, no detectable phosphorous was found in pituitary bST prepared by the method described here, as shown in Fig. 6. Based on a phosphoserine standard, we estimate that there could be no more than 4 μg of phosphoserine in the 175-mg pituitary bST sample that was examined.

Biological Activity of Pituitary bST—Having established that the pituitary bST preparation contained both the expected amino acid sequence variants at the NH2 terminus and at position 126 as well as a postulated isooxosapartate derivative at position 128, the effect of these variations upon biological activity was investigated. Fig. 7 shows the results of a bovine liver receptor-binding assay of pituitary bST and methionyl-bST. Both proteins showed identical binding affinity for bovine liver receptors in the assay. None of the sequence or structural differences between the pituitary and recombinant preparations had an apparent effect upon this measure of somatotropin activity.

The protein preparations were also compared in an in vivo assay in which weight gain in mature female rats was determined. As shown in Fig. 8, no statistically significant differences in potency were observed between methionyl-bST and pituitary-derived somatotropin.

Previous studies of the galactopoietic response to somatotropin in dairy cattle (21) have demonstrated that NIH standard lots of bST obtained from A. Parlow displayed lower milk production activity than any of several recombinant somatotropins, including the Met1-Leu126, Met1-Val126, Ala1-Leu126 bST, and Ala1-Val126 bST variants. However, the Parlow bST preparations were subsequently shown by NH2-terminal sequence analysis to contain 40–50% des(1–4) molecules, as shown in Table II. In a subsequent study (22), recombinant des(1–4) somatotropin (NH2-terminal sequence 20% Met-Ser-Leu-Ser—80% Ser-Leu-Ser) was purified, assayed for galactopoietic activity, and also found to have decreased activity relative to full length recombinant bST.

**DISCUSSION**

A purification procedure for bovine somatotropin from pituitaries has been developed which results in protein of high
purity with 2–4-fold greater yield than methods reported previously. This result may be due to the enhanced solubility of somatotropin in urea-containing buffers and correspondingly better recovery of bST in the initial pituitary extract.

When purified bST was analyzed for protein purity by the methods of SDS-PAGE and Western blotting, the preparation was determined to be greater than 98% pure. Reversed-phase HPLC analysis can sometimes be used as a criterion of protein purity; and in the case of pituitary bST, this analysis served to identify heterogeneity in the preparation. As shown in Fig. 1, at least three protein species are resolved. However, the data in Fig. 2 suggested that all of these HPLC peaks are bST isoforms. A probable source of the heterogeneity which generates peaks 3 and 4 in Fig. 1 appears to be the allelic variation at position 126. The observed retention times on RP-HPLC for recombinant somatotropins containing either Leu126 or Val126 were consistent with Val126 bST as the earlier eluting peak 3 and Leu126 bST as the isoform contained in peak 4. Besides providing a high resolution analysis for bST isoforms, these data have shown that purified bST produced by this procedure is free of contaminating protein within detection limits of the methods employed.

The procedure described above contained relatively few steps and conceivably could be scaled up for production of 10–20-g quantities. The incorporation of a filtration step prior to loading the DEAE-cellulose column would be necessary to remove particulates. Batch treatment of the pituitary extract with DEAE-cellulose or DEAE-dextran precipitation to remove the bulk of soluble pituitary proteins prior to chromatography would also be advantageous. Diafiltration equipment could be substituted for the G-25 buffer exchange step prior to CM-cellulose chromatography. If the yield values shown in Table I proved to be directly scalable, 10 g of bST could be obtained from 650 pituitary glands.

One goal of pituitary bST characterization has been to identify naturally occurring microheterogeneity in the protein and to determine whether the microheterogeneity has significance for biological function. To achieve this goal, we have isolated the protein under mild conditions that would not generate chemical modifications. Of equal importance is the need to isolate all of the isoforms that may be present in the tissue. If somatotropin species were present in vivo which differed slightly in charge, molecular weight, conformation, or some other chemical property, they might be selectively removed during the purification process. Our approach to ascertain that no bST species were being lost during purification was to perform a two-dimensional electrophoretic separation of the original pituitary extract, probe the gel for bST species by Western blot analysis using a polyclonal antiserum, and then perform the same analysis on the purified bST to determine that the same Western blot pattern was obtained.

The data in Fig. 4 strongly argue that this goal has been achieved, at least within the detection limits of our analytical methods. Quantitation of relative protein mass between the extract and the purified material was by silver-staining intensity, whereas the more sensitive Western blotting procedure was able to identify bST species present qualitatively. Western blot methods have been subject to variability in protein transfer, antibody recognition, and staining of different protein species, and therefore do not lend themselves readily to quantitation. In the Western blot of the pituitary extract (Fig. 4c), trace amounts of acidic bST species were detected which were not present in purified material (Fig. 4d). However, these species were not a significant part of the protein mass, as indicated by silver-staining (Fig. 4a). Although this method of preparation does appear to yield purified bST that contains those species present in the extract, we cannot yet definitively rule out the possibility that additional somatotropin species may exist in vivo which were not present in the original extract.

In addition to electrophoretic analysis, a variety of high resolution chemical analyses was applied to the purified protein in order to confirm isoforms identified previously and to attempt to discover isoforms undetected previously. NH2-terminal sequence analysis confirmed previous reports of heterogeneity, in which roughly half of the secreted bST molecules lack alanine as the NH2-terminal residue. Significantly, none of the des(1–4) variant was detected in this preparation, strongly suggesting that it is not a natural component of pituitary bST.

The Met-Ser-Leu-Gly- and Ser-Leu-Ser-Gly-NH2-terminal deletion variants have been shown by this (see Fig. 4, e and f) and previous studies to be an artifact of isolation, due most likely to a proteolytic activity with a pH optimum between 5 and 6.5 (2, 23). The present purification has avoided this potential degradation by having performed the initial extraction and chromatography steps at alkaline pH.

Tryptic mapping by reversed-phase HPLC has proven to be a method of extremely high resolution when applied to bST and has suggested the presence of an isoform undetected previously. A tryptic peptide was identified which comprised 20% of the protein mass and which was not present in a recombinant somatotropin produced by heterologous gene expression in E. coli. The presence of an Asp-Gly sequence in the peptide coupled with the loss of signal from the gas-phase sequenator has suggested the presence of an aspartic acid to isoaspartic rearrangement in the protein. Rearrangement through a succinimidyl ring intermediate in peptides containing similar sequences has been shown to occur under conditions of elevated temperature to yield isoaspartic acid and the continuation of the peptide chain through the side chain δ-carboxyl group (16).

Studies of the rearrangement reaction in model peptides have shown that an aspartate to isoaspartate rearrangement
should not occur at an appreciable rate under the conditions used in this paper for isolation of bST from pituitary (16). In studies of carboxymethyltransferase activity using glucagon as a substrate, Ota et al. (24) determined that base treatment of glucagon (0.1 m NH₄OH, pH 10.1, 37 °C, 3 h) resulted in rearrangement to L-isoaspartate at some asparagine residues but not at aspartate residues. Therefore, the conditions of 50 mM Tris base, pH 10.3, 4 °C used in the DEAE-cellulose chromatography step in this preparation of pituitary bST appear unlikely to have yielded the observed level of isoaspartate.

These data suggest that the Asp¹²⁸ β-linked bST protein may be a component of somatotropin in vivo. The rate of isoaspartate formation in vivo has not been measured directly, but model peptide studies of rearrangement at an Asp-Gly sequence (Asp¹²⁸) in bST is also followed by glycine) suggest that the rate is slow at physiological temperature and pH, with a t₀ on the order of weeks (15). However, the rate of reaction in proteins at a given aspartate residue is probably a function of tertiary structure as well as sequence (24, 25). Carboxymethyltransferase activity, which has been proposed to be involved in repair of isoaspartyl damage in proteins by restoring the peptide bond (26), may have a role in determining the observed level of isoaspartate in bovine somatotropin. This activity has been isolated from the cytosolic fraction of pituitary tissue, although it is not yet known whether the enzyme is present in secretory granules harboring bST (27). The observed level of Asp¹²⁸ derivatization in pituitary bST may prove to be a complicated function of the rates of isomerization, carboxymethyltransferase activity, and secretion.

Our analysis of pituitary bST for phosphorylated amino acids using ³¹P NMR has demonstrated conclusively that the preparation contains no significant levels of phosphorus. This result is in contrast to a previous report that phosphate esters were present in pituitary ovine somatotropin (20). The mild conditions used for isolation in the method described herein have eliminated the possibility that phosphorus was lost during purification. The possibility exists that phosphorylation is specific to the ovine system and is not present in bovine, although this seems unlikely. More probably, the presence of phosphorylation in the ovine preparation was due to nonspecific kinase activity encountered during purification, which was avoided by the method described in this report.

To assess the effect of pituitary bST microheterogeneity on activity, both direct biadiing studies to receptors in bovine liver membranes and growth response in a rat growth model were employed. For both assays, pituitary bST and recombinant somatotropin were found to have equivalent specific activities, as shown in Figs. 7 and 8. Similar conclusions regarding biological activity have been reached by other investigators comparing recombinant bovine somatotropin with pituitary bST obtained from NIH through the National Hormone and Pituitary Program (U. S. A.) (28).

In addition to demonstrating the equal potency of recombinant and pituitary bST, the activity assay results have suggested that the proposed β-linked component at Asp¹²⁸ contains substantial activity. If this component comprising 20% of the protein mass were totally inactive, the decrease would have been detectable by the rat growth assay.

The rat growth assay employed has been shown to correlate with decreased galactopoietic activity in dairy cows (22) and therefore appears to be a valid predictor of milk yield for bST variants less active than methionyl-bST. Although the pituitary bST preparation described here has not yet been assayed directly for galactopoietic activity, we postulate that full activity would be observed. The decreased activity of the NIH standard lot of pituitary bST obtained from A. Parlow relative to recombinant methionyl-bST in the earlier studies (21) was due most likely to the high level of NH₂-terminal deletion variants present in the Parlow preparation (see Table II).

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