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Short communication

Matrix-assisted laser desorption mass spectrometry for the detection of recombinant bovine growth hormone in sustained-release form

Vitaliano Borromeo[a], Anna Berrini[a], Camillo Secchi[a,*], Gian Franco Brambilla[b], Alfredo Cantafora[b]

[a]*Istituto di Fisiologia Veterinaria e Biochimica, Via Celoria 10, I-20133 Milan, Italy*
[b]*Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Rome, Italy*

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Abstract

We employed matrix-assisted laser desorption mass spectrometry (LD-MS) to detect recombinant bovine growth hormone (r-bGH) in sustained-release preparations. After preliminary extraction in phosphate buffer, LD-MS provided a precise determination of the molecular mass (M_r) of the r-bGH contained in 38 sustained-release preparations. The hormone was characterised using enzyme immunoassay, immunoblotting and amino acid sequencing. Rapid detection is essential for analysing large numbers of samples, and for monitoring the use of r-bGH in zootechnical productions and its administration as a "high-tech" drug for therapeutic purposes.

1. Introduction

Recent advances in matrix-assisted laser desorption mass spectrometry (LD-MS) have provided a versatile analytical tool for the characterisation of proteins, glycoproteins, carbohydrates and other biomolecules. LD-MS rapidly and accurately measures the molecular mass (M_r) of intact proteins, and the method is sensitive enough for the characterisation of protein transcriptional variants and co- and post-translational modifications. Recombinant biotechnology-derived proteins, such as bovine growth hormone (bGH), can also be studied. Various forms of recombinant bGH (r-bGH) with slightly different -NH_2 terminal sequences have been produced, and described in the literature [1,2].

Exogenous r-bGH is given to dairy cattle to exploit the hormone's galactopoietic effect. The safety of the milk produced by r-bGH-treated cows is well attested, but the possible side-effects on animal welfare are still open to discussion [3].

Formulation approaches for r-bGH have been driven primarily by market needs for a sustained-release preparation; the most common formulations involve the dispersion of r-bGH in low-cost oils or waxes, that lead to elevated plasma levels in treated cows for one to four weeks [4].

The use of r-bGH is not allowed in the EC,

* Corresponding author.

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former Czechoslovakia, Israel and the USA have licensed the administration to dairy cattle. This parallel market of veterinary drugs may be a source of uncontrolled distribution of r-bGH toward the EC. This prompted us to set up a fast method for the detection and characterisation of the r-bGH molecules in sustained-release formulations.

2. Experimental

2.1. Hormones

The pituitary hormone (p-bGH) was purified from pituitary tissue as previously described [5]. A lyophilised standard recombinant hormone (r-bGH) was kindly provided by Eli Lilly (Eli Lilly Italy, Sesto Fiorentino, Firenze, Italy) (product supplied to C.S. for laboratory analyses). The recombinant hormone had an additional octapeptide at its N-terminal end (M-F-P-L-D-D-D-D) and the first residue of the p-bGH (Ala) was replaced with a Lys [6]. In addition, the recombinant molecule lacks the variant Val/Leu at position 127, but the molecule showed only Leu_{127}. The M_r of the two hormones, calculated from the amino acid sequence, was 21 819 for the p-bGH and 22 825 for the r-bGH.

2.2. Extraction of long-acting preparations

Thirty-eight plastic syringes fitted with needles, containing about 3 g of a sustained-release preparation, provided by the Italian Ministry of Health, were tested. The syringes had no identification. Fifty milligrams of the product in each syringe were dissolved in a conical vial in 5 ml of 10 m*M* phosphate buffer, 0.15 *M* NaCl pH 7.4 (PBS). After vortex-mixing, sonication (5 min) and centrifugation (3000 *g*, 15 min), the supernatant was collected and stored at −20°C until analysed.

A second 50-mg aliquot of the same samples was similarly extracted in the presence of 1% sodium dodecyl sulphate (SDS). The fractions extracted in the presence of SDS were further purified by reversed-phase liquid chromatography (HPLC). An HPLC System Gold was coupled with a Model 168 detector (Beckman Analytical, Stanford Industrial Park, CA, USA). The column used was a LiChrospher 100 RP-18 (5 μm) 250 × 4 mm I.D. (Merck, Darmstadt, Germany) and was operated at a flow-rate of 0.5 ml/min in *n*-propanol–0.017 *M* H_3PO_4 brought to pH 4.0 with diethylamine. The eluate was recorded at 224 and 280 nm. The sample was eluted with a 20-min linear gradient from 40 to 80% of the organic phase. The recovered peak was evaporated nearly to dryness under a stream of nitrogen, and the residue was dissolved in water and stored at −20°C until analysed.

2.3. Matrix-assisted laser desorption mass spectrometry

M_r values were obtained by LD-MS using a Laser Mat (Finnigan Mat, Hemel Hempstead, UK). Light from a pulsed nitrogen laser (337 nm) was focused onto the sample target at a power density around 106 W/cm^2. The ions desorbed by each laser pulse were accelerated to 20 keV energy along a 0.5-m time-of-flight (TOF) drift tube. Typically, the spectra were taken by adding together ten single-shot spectra at a laser flunk just above the threshold for ion production. Calibration was performed as suggested by the instrument manufacturer, with a low M_r oligosaccharide dextran. The m/z values of the samples were calculated from this curve by an external standard calibration method. The mass accuracy, against human insulin as a standard protein, was ± 0.3%.

2.4. Enzyme immunoassay

The homologue enzyme immunoassay (EIA) used for bGH was as previously described [7]. The assay has a detection limit of 0.25 ng/ml; the intra-assay coefficient of variation (C.V.) ranged from 2.6 to 5.1% and the inter-assay C.V. from 8.5 to 12.7%. Standard r-bGH shows 77.23% cross-reactivity with the standard p-bGH [4].

2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-electrophoresis was done in polyacrylamide gradient gel (7–20%) overlaid with 4% stacking gel using a Minigel apparatus (Biometra, Göttingen, Germany) [8]. The bGH concentration of samples was established by EIA. An amount of each sample containing 4 μg bGH was dissolved with 2.5% SDS and 5% 2-mercaptoethanol and loaded in two different gels. After electrophoresis, one gel was fixed and stained with Coomassie Brilliant Blue R-250, and the second was electroblotted onto a PVDF sheet (Immobilon P, Millipore, Milan, Italy) using a semi-dry apparatus (Biometra, Göttingen, Germany). The bands corresponding to bGH were detected using rabbit anti-bGH polyclonal antibodies followed by peroxidase-conjugated anti-rabbit immunoglobulins. Immunoperoxidase staining was done as described by Towbin et al. [9].

2.6. Amino acid sequence

A sample of 3 nmol of HPLC purified r-bGH was submitted to ten cycles of analysis using a 477A automatic protein sequencer (Applied Biosystems, Milan, Italy).

3. Results and discussion

Fig. 1 reports the LD-MS spectra of the standard p-bGH and r-bGH. In addition to the characteristic single protonated protein ion $(MH)^+$ (M_r 21 783 for the p-bGH and M_r 22 872 for the r-bGH) and the double protonated ion $(MH_2)^{2+}$ (M_r 10 919 and M_r 11 454, respectively), both spectra showed peaks corresponding to polymeric forms. Only the dimeric, single ionised form $(M_2H)^+$ was considered because the intensity of ionised aggregates rapidly decreased as the number of monomer units increased. Under the working conditions used, dimers, trimers and tetramers, present in both the p- and r-bGH, amounted to about 1/3, 1/9 and 1/27 of monomer intensity. Generally the trimer was only just evident, and the tetramer got confused in the noise.

The M_r of the standard p- and r-bGH acquired by LD-MS differed by less than 0.3% from the hormonal M_r values calculated from the sequences (21 819 for the p-bGH and 22 825 for the r-bGH).

To detect the bGH in sustained-release preparations it was necessary to extract the hormone from the waxes and oils added. Fig. 2 shows the LD-MS spectrum of a PBS extract. LD-MS analyses gave an accurate M_r determination of the r-bGH dispersed in the sustained-release preparations. The spectrum presented peaks at the same M_r as the standard r-bGH. The peak at M_r 22 898 corresponded to the molecular ion, and the three additional peaks at M_r 45 772, M_r 11 465 and M_r 7650 corresponded respectively to the single ionised dimer, and to the double and triple protonated monomer. All the sustained-release preparations assayed gave similar spectra. The peak corresponding to the molecular ion showed a mean M_r ($\pm$ S.D.) of 22 883 $\pm$ 33 (n = 38).

The high M_r forms observed in both standard and extracted bGH mass spectra, resulted from the aggregation of molecules. Higher M_r variants of bGH have been observed in many preparations of the hormone and appear to be related to its treatment and storage; they show lower potency in radioimmunoassay and radioreceptor assay [10].

To increase the recovery of immunoactive bGH, we tried extraction in PBS with SDS. The addition of SDS increased the bGH immunological activity recovered from 19.2 $\pm$ 3.1 to 96.0 $\pm$ 5.5 mg of immunoactive bGH per gram of product (mean $\pm$ S.D.); the presence of SDS did not affect the EIA, but is not recommended for LD-MS analysis since detergents tend to disperse over the surface of the sample, interfering with laser energy uptake by the matrix–sample complex and its further evaporation [11].

In order to overcome this, an HPLC run was done but we only recovered about 1/3 of the loaded immunoactive bGH (28.7 $\pm$ 4.1 mg of immunoactive bGH/g of product). This low immunological activity recovered was not neces-

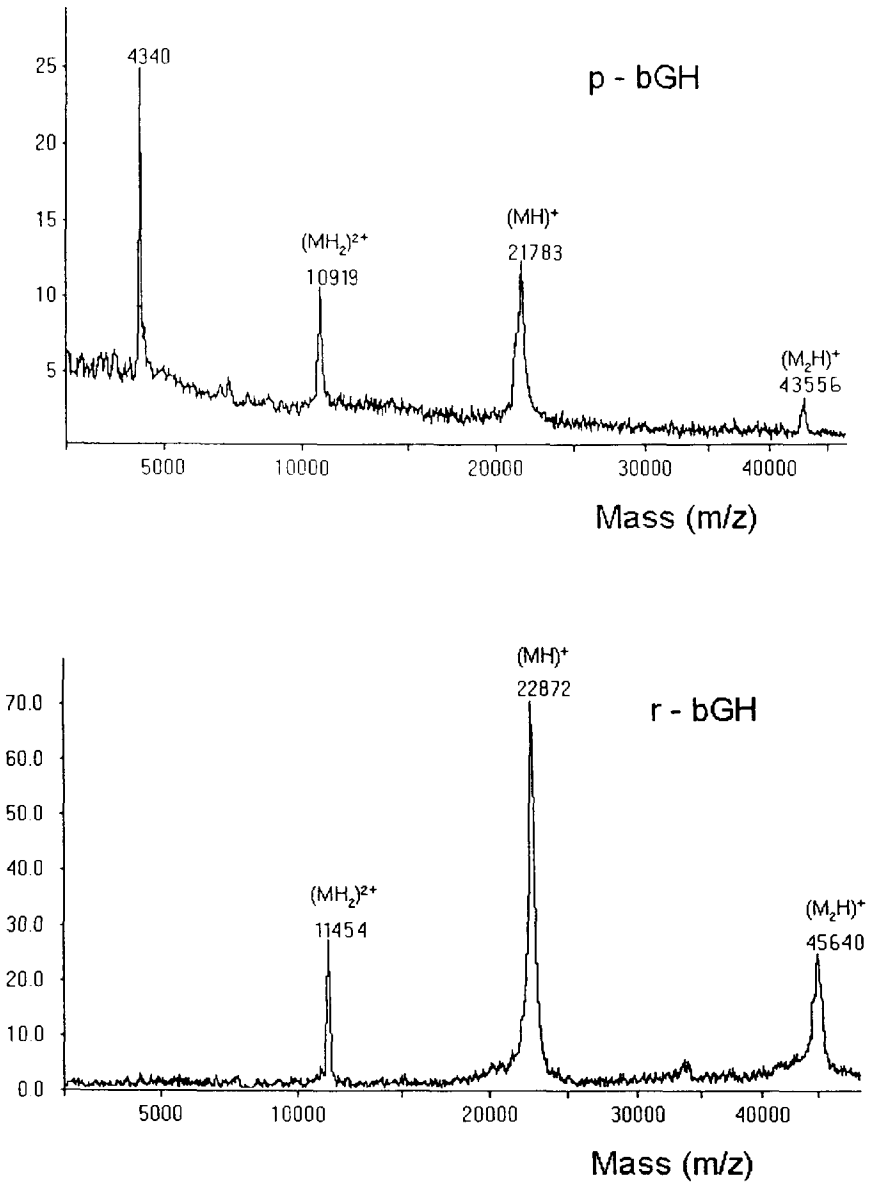

Fig. 1. LD-MS spectra of the p-bGH and r-bGH used as standards. The presence of either multiple ionisation or aggregate forms is indicated as follows: $(M_2H)^+$ = dimer, single ionisation; $(MH_2)^{2+}$ = monomer, double ionisation; $(MH_3)^{3+}$ = monomer, triple ionisation.

sarily due to a loss of bGH molecules, but the HPLC separation was carried out at low pH (pH 4.0) and in the presence of organic solvents. These harsh conditions might modify the bGH epitopes recognised by the antiserum, and lower the bGH immunological reactivity [11].

The fractions extracted without SDS and the HPLC purified fractions gave similar spectra, although the HPLC fraction had a lower background on account of the higher hormonal content and the lower concentration of buffer salts.

These results confirm the specificity of the

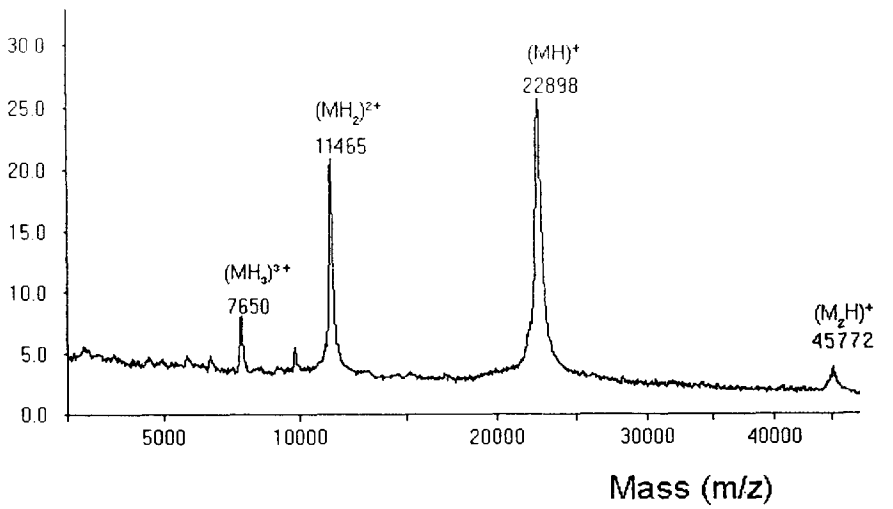

Fig. 2. LD-MS spectrum of the fraction from a sustained-release preparation extracted with PBS as described in the text. The presence of either multiple ionisation or aggregate forms is indicated as follows: $(M_2H)^+$ = dimer, single ionisation; $(MH_2)^{2+}$ = monomer, double ionisation; $(MH_3)^{3+}$ = monomer, triple ionisation.

simple PBS extraction. On the other hand, the slightly higher recovery of the immunological activity with SDS-HPLC does not counterbalance the increased time and handling complexity of the whole alternative protocol. In view of the rapidity and simplicity of the method, the most favourable extraction procedure is without SDS.

In order to better characterise the homogeneity of the extracted products, an SDS-electrophoresis followed by immunoblotting was carried out. Fig. 3A shows the SDS-electrophoretic pattern of standard p- and r-bGH, and of representative PBS-extracted and HPLC-purified fractions. Both the extracted (lane 4) and the HPLC (lane 3) fractions showed a major band with the same electrophoretic mobility as the standard r-bGH (lane 2); these bands showed an apparent M_r of 23 450, determined by comparison of the band position relative to standard molecular masses. A second faint band with M_r = 48 600 was present in the pattern of the HPLC fraction; this was a dimeric aggregation form of the r-bGH. The p-bGH (lane 1) shows higher electrophoretic mobility with an apparent M_r = 20 700.

After electroblotting onto the PVDF sheet, all the bands reacted with the anti-bGH polyclonal antibodies (Fig 3B). The HPLC fraction showed other bands with higher M_r reacting with the anti-bGH polyclonal antibodies. The calculated M_r of these bands corresponded to polymeric forms of the monomeric hormone. As discussed before for MS–LD analyses, higher M_r variants of GH are frequently observed in many preparations of the hormone. The strong separating conditions used in HPLC probably caused this enrichment.

The first ten NH_2-terminal aminoacidic residues of the HPLC-purified r-bGH obtained from sequence analysis were NH_2-Met-Phe-Pro-Leu-Asp-Asp-Asp-Asp-Lys-Phe, indicating that the r-bGH found in the preparations is structurally the same as the standard [6].

In conclusion, the coupling of EIA with LD-MS can be exploited to detect immunoreactive bGH and establish its M_r on primary extracts of sustained-release preparations. The LD-MS technique is very rapid (analysis time less than 5 min) and suitable for routine analysis. Rapid detection is fundamental in checking risks and benefits on animal health and consumer safety arising from the use of r-bGH as "high-tech" drug in zootechnical production [12].

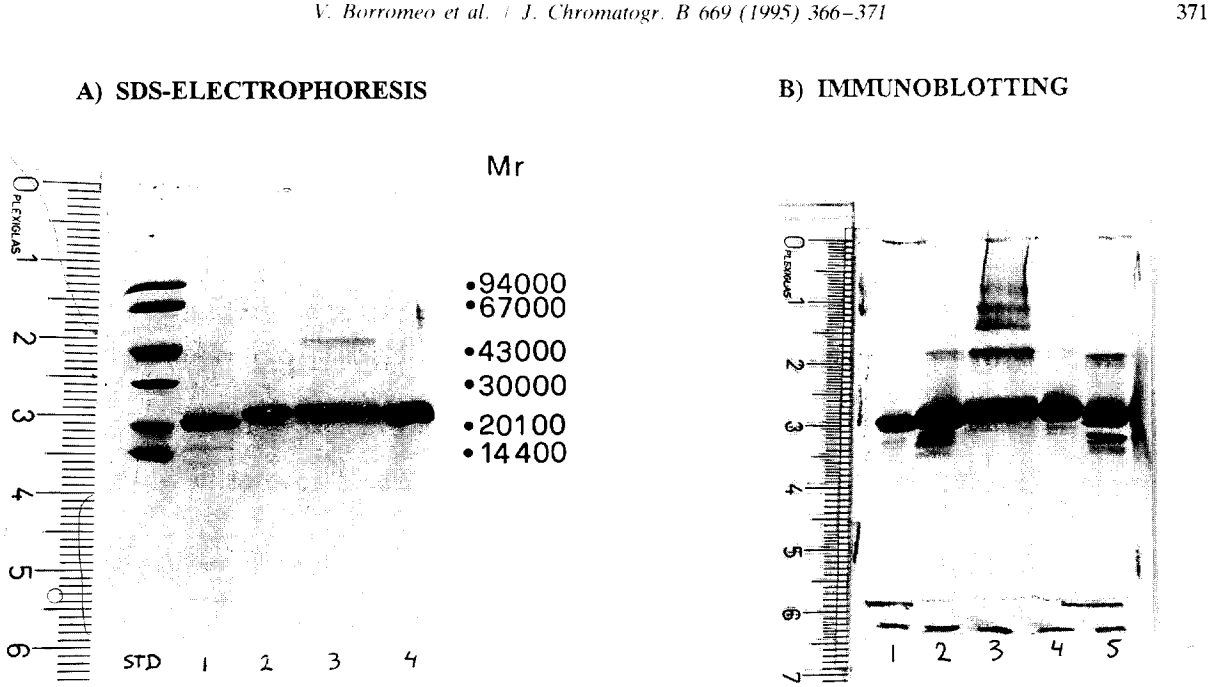

Fig. 3. SDS-electrophoresis and electroblotting of standard p- and r-bGH and of representative fractions extracted and purified by HPLC from sustained-release preparations. (A) SDS-electrophoresis in polyacrylamide gradient gel. STD = standard proteins for molecular mass; 1 = standard p-bGH; 2 = standard r-bGH; 3 = HPLC purified fraction; 4 = fraction extracted from sustained-release preparation. (B) Immunoperoxidase stain of the PVDF blotted membrane after incubation with rabbit anti-bGH polyclonal antibodies and peroxidase-conjugated anti-rabbit immunoglobulins: 1 and 4 = standard r-bGH; 2 = fraction extracted from sustained-release preparation; 3 = HPLC purified fraction; 5 = standard p-bGH.

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