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1 # Production of polyclonal antibodies directed to recombinant methionyl bovine
2 # somatotropin HIGHLIGHTED

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29 **Abstract**

30 The administration of recombinant methionyl bovine somatotropin (rMbST) to dairy cows to
31 increase milk yield remains a common practice in many countries including the USA, Brazil,
32 Mexico, South Africa and Korea, whereas it has been forbidden within the European Union
33 (EU) since 1999. A rapid screening immunoanalytical method capable of the unequivocal
34 determination of rMbST in milk would be highly desirable in order to effectively monitor
35 compliance with the EU-wide ban for home-made or imported dairy products. For decades,
36 the production of specific antibodies for this recombinant isoform of bovine somatotropin
37 (bST) has remained elusive, due to the high degree of sequence homology between both
38 counterparts (e.g. methionine for rMbST in substitution of alanine in bST at the *N*-terminus).
39 In this study, we compared several immunizing strategies for the production of specific
40 polyclonal antibodies (pAbs), based on the use of the full-length recombinant protein, an
41 rMbST *N*-terminus peptide fragment and a multiple antigen peptide (MAP) which consists of
42 an oligomeric branching lysine core attached to the first two *N*-terminus amino acids of
43 rMbST, methionine and phenylalanine (MF-MAP). The immunization with KLH-conjugated
44 MF-MAP led to the production of the pAb with the highest rMbST/bST recognition ratio
45 amongst the generated battery of antibodies. The pAb exhibited a specific binding ability to
46 rMbST in a competitive antigen-coated ELISA format, which avidity was further improved

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47 after purification by rMbST *N*-terminus peptide-based affinity chromatography. These results
48 suggest that immunodiscrimination between structurally related proteins can be achieved
49 using immuno-enhanced immunogens such as MAPs.
50
51 **Keywords**: recombinant methionyl bovine somatotropin, polyclonal antibodies,
52 immunodiscrimination, multiple antigen peptide.
53
54 **Abbreviations**: bST, bovine somatotropin; rMbST, recombinant methionyl bovine
55 somatotropin; MAP, multiple antigen peptide; pAb, polyclonal antibody.
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57 **1. Introduction**

58 Bovine somatotropin (bST), with the majority isoform of 191-amino acid protein and a
59 molecular weight of 21802 Daltons (Table 1), is produced and secreted by the anterior
60 pituitary gland. Through a complex network system, the somatotropic axis, this hormone
61 regulates several physiological processes involved in metabolism, growth and reproduction
62 [1]. It is well documented that the exogenous administration of bST redirects nutritional
63 partitioning towards milk synthesis in dairy cows, which is therefore translated into an
64 increase in milk production ranging from 10 to 40% [2,3]. Genetically-engineered or
65 recombinant isoforms of the bovine somatotropin have been developed and produced since
66 the early 1990's. Recombinant methionyl bST (rMbST; Table 1), initially commercialized by
67 Monsanto and then by Elanco (Animal Division of Eli Lilly and Company) under the trade
68 name of Posilac®, is the only commercial product approved by the Food and Drug
69 Administration (FDA) in the USA and by the corresponding competent authorities in Brazil,
70 Mexico, South Africa and Korea. However, its marketing and utilization as well as the trade
71 of dairy products obtained from rMbST-treated animals, are prohibited within the EU [4], and
72 other countries such as Japan, China, Australia, New Zealand or Canada. Along with the on-
73 going hormone debates between the EU and the USA, controversy has surrounded rMbST
74 since it became commercially available in 1994, with growing concern about the implications
75 of the administration of this synthetic protein on human and animal health and welfare.
76 Several adverse effects reported for treated animals include diminished fertility and an
77 increased occurrence of lameness and clinical mastitis [5, 6], which requires additional
78 antibiotic treatments that may cause further food safety concern regarding antibiotic residues
79 in dairy and other food products. Extensive use of antibiotics in modern agricultural farming
80 has also been linked to the development and emergence of antibiotic resistance that is
81 currently affecting both human and veterinary medicine worldwide [7]. Moreover,

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82 administration of somatotropin raises the concentration of Insulin-like Growth Factor 1 (IGF-
83 1) in milk [8]. Elevated circulating levels of IGF-1 have been associated with a higher risk of
84 developing several types of cancer [9, 10]. However, studies correlating the intake of milk
85 from rMbST-treated animals with human diseases are still lacking.
86 In order to control illegal administration of rMbST and to ensure high quality and safety of
87 milk and consumer protection, reliable analytical methodologies capable of unambiguous
88 identification of the synthetic methionyl growth hormone in milk are required. Current
89 analytical methods for determination of rMbST rely on instrumental technologies such as
90 HPLC-MS/MS [11-14]. Despite the fact that chromatographic systems are highly sensitive
91 and specific, these techniques often limit their applications for rapid screening of a large
92 number of samples due to the requirement of extensive sample preparation time and
93 sophisticated instrumentation which is also laboratory-based. Immunoanalytical methods, in
94 particular Enzyme-Linked Immunosorbent Assays (ELISA), are widely used as rapid
95 screening tools for routine monitoring of food contaminants and residues, owing to their
96 simplicity, cost-effectiveness and capabilities of performing high-throughput analysis. Two
97 different immunoanalytical approaches have been adopted for the detection of rMbST. By the
98 direct strategy, the presence of the native and the recombinant isoforms is determined
99 simultaneously in biological fluids [15-17], whereas the indirect approach is based on the
100 analysis of biomarkers of which their concentration is increased upon rMbST administration.
101 IGF-1 has been the traditional target measured for this purpose [8, 17-20]. Methods based on
102 the detection of anti-rMbST immunoglobulins in treated cows have also been published [21,
103 22]. Nevertheless, the direct analysis of rMbST itself is highly preferable, in order to
104 circumvent problems associated with inter and intra-individual variation of biomarkers
105 expression levels, which can lead to misinterpretation of results.

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106 To date, no specific immunoassays have been described for the detection of rMbST. The high
107 sequence homology displayed by the recombinant and the native somatotropins (methionine
108 in substitution of alanine at the *N*-terminus) has greatly hindered the successful production of
109 rMbST-selective antibodies. The strategy most often used entails the immunization with the
110 complete recombinant protein [21, 23-25]. Nevertheless, the antibodies produced following
111 this procedure have generally failed in their capacity to immunodiscriminate between bST
112 and rMbST, while a 2–fold increased affinity factor towards rMbST was described for the
113 mAb-based sandwich assay developed by Erhard *et al* [23]. Considering that only one amino
114 acid of difference at the *N*-terminus is encountered, the immunization with the whole protein
115 most likely leads to the production of antibodies directed towards shared epitopes in both
116 counterparts, bST and rMbST, therefore being unable to specifically recognize the latter. On
117 the other hand, a frequently accomplished practice for raising antibodies against proteins is
118 based on the use of immunizing synthetic peptide fragments which mimic concrete sequences
119 within the target [26-28]. The immunization with a synthetic peptide representing the
120 differential *N*-terminus of rMbST could *a priori* focus the immune response towards the
121 recognition of the characteristic epitope of the protein. Castigliego *et al* described for the first
122 time the production of a mAb by using a synthetic nine amino acid rMbST *N*-terminus-
123 mimicking peptide coupled to KLH as immunogen [16]. Despite showing a 3–fold higher
124 affinity towards rMbST than to bST, complete immunodiscrimination was not yet possible by
125 using the developed immunoassays. As an alternative to monovalent peptides, multiple
126 antigen peptides (MAPs) or multimerized peptides, have been used as immunogens since
127 they were developed in 1988 [29], especially in the area of vaccine development [30-32].
128 MAPs have been shown to efficiently improve the immunogenicity of a particular antigen,
129 thus eliciting a stronger immune response, as a consequence of the presentation of multiple
130 copies to the immune system [33]. Furthermore, it has been reported that the resulting

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131 immune response is generally mono-specific and more homogeneous [34]. The production of
132 pAbs targeted at vertebrate somatotropins using a synthetic MAP constituted by several
133 copies of an 18 amino acid highly conserved domain proximal to the *C*-terminus of the
134 protein has already been published [35]. To our knowledge this system has never been
135 applied before for the production of rMbST-specific antibodies.

136 In the present work several immunization strategies have been compared for the production
137 of anti-rMbST rabbit pAbs. The immunization with an octavalent synthetic rMbST *N*-
138 terminus dipeptide-mimicking MAP, followed by affinity purification with a synthetic
139 rMbST *N*-terminus-mimicking linear peptide, resulted in the production of a pAb capable of
140 specifically differentiating between the recombinant and the native somatotropins in a
141 competitive ELISA format.

142 2. Experimental

143 2.1. Reagents and instrumentation

144 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(*N*-morpholino)ethanesulfonic acid,
145 2-(4-morpholino)ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl)
146 carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Aldrich
147 (St. Louis, MO, USA). Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate
148 (sulfo-SMCC) was acquired from Thermo Fisher Scientific (Rockford, IL, USA). Slide-A-
149 Lyzer dialysis cassettes and Zeba 7kDa Desalting columns from Thermo Fisher Scientific
150 (Rockford, IL, USA) were used for the purification of the immunizing conjugates. Keyhole
151 limpet hemocyanin (KLH) carrier protein, complete and incomplete Freund's Adjuvants, the
152 bicinchoninic acid (BCA) test kit were from Sigma Aldrich (St. Louis, MO, USA). rMbST *N*-
153 terminus dipeptide-mimicking MAP, MF-MAP-C (Figure 1), was acquired from GenScript

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154 (Piscataway, NJ, USA). Synthetic rMbST *N*-terminus-mimicking linear peptides EP091213
155 (amino acid sequence: H_2N–Met-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu-Phe-Ala-Asn-Ala-
156 Val-Leu-Arg-Cys–COOH) and EP093536 (amino acid sequence: H_2N–Met-Phe-Pro-Ala-
157 Met-Ser-Leu-Ser-Gly-Leu-Phe-Cys–$CONH_2$), used for animal immunization and antibody
158 purification, respectively, were purchased from Eurogentec S.A. (Seraing, Belgium). Affinity
159 columns packed with Protein A Sepharose 4 Fast Flow gel purchased from GE Healthcare
160 were used for the purification of rabbit antibodies. EP093536 peptide affinity column was
161 prepared using Toyopearl AF-Amino-650M gel from Tosoh Bioscience GmbH (Stuttgart,
162 Germany). Maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) and streptavidin
163 poly−HRP (STV−pHRP) were acquired from Thermo Fisher Scientific (Rockford, IL, USA).
164 Centricon Plus-20 ultracentrifugation concentration devices (molecular cut-off 3K) were
165 purchased from Millipore (Billerica, MA, USA). Peroxidase-conjugated polyclonal goat anti-
166 rabbit immunoglobulin (GAR−HRP) was purchased from Sigma Aldrich. Biotin-SP-
167 conjugated polyclonal goat anti-rabbit IgG (H+L) (GAR−b) was obtained from Jackson
168 Immunoresearch Europe (Suffolk, United Kingdom). Gelatin was from Merck (Darmstadt,
169 Germany). Bovine serum albumin (BSA) was from Sigma Aldrich. Lactoferrin from bovine
170 milk was provided by Taradon Laboratory SPRL (Tubize, Belgium). β-lactoglobulin from
171 bovine milk was from Sigma Aldrich. Casein from bovine milk was purchased from Merck.
172 Bovine prolactin was from the National Institute of Health (NIH, USA). Bovine placental
173 lactogen was provided by Jean-François Beckers from the University of Liège (Liège,
174 Belgium). Pepstatin, EDTA and acetic acid were from Sigma Aldrich (St. Louis, MO, USA).
175 Sequencing-grade modified trypsin (EC 3.4.21.4) was from Promega (Madison, WI, USA).
176 Ninety-six-well flat-bottom Nunc Maxisorp polystyrene ELISA plates were purchased from
177 Nunc (Roskilde, Denmark). ELISA plate washer model 1575 Immunowash was from Bio-
178 Rad Laboratories (Hercules, CA, USA). 3,3',5,5'-Tetramethylbenzidine/H_2O_2 solution was

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179 from BioFX Laboratories (Owings Mills, MD, USA). ELISA absorbance was monitored at
180 450 nm using a Multiskan EX reader provided by Thermo Fisher Scientific (Zellik, Belgium).
181 Buffers and solutions: *1) Coating buffer (CB)*: 50 mM sodium carbonate–bicarbonate buffer
182 (pH 9.6). *2) Blocking solution*: CB containing 0.5% (w/v) gelatin. *3) EIA buffer*: 5.6 mM
183 $Na_2HPO_4 \cdot 2H_2O$, 0.9 mM $NaH_2PO_4 \cdot 2H_2O$, 150 mM NaCl, (pH 7.4), containing 0.02% (w/v)
184 gelatin, 0.005% (v/v) Tween 20, 0.001% (w/v) 8-Anilino-1-naphthalenesulfonic acid
185 ammonium salt, 0.005% (w/v) ascorbic acid, 0.01% (w/v) thimerosal. *4) Washing solution*:
186 150 mM NaCl, containing 0.05% (v/v) Tween 20. *5) PBS*: 44 mM sodium phosphate, 6 mM
187 potassium phosphate, 154 mM NaCl, (pH 7.4). *6) PBST*: PBS containing 0.05% (v/v) Tween
188 20.

189 **2.2. Somatotropin standards**

190 Pituitary bovine somatotropin (bST) was purchased from the National Hormone and Peptide
191 Program (NHPP), Harbor-UCLA Medical Centre (Torrance, CA, USA). Pituitary porcine
192 somatotropin (pST) was from Sigma Aldrich. Recombinant methionyl equine somatotropin
193 (rMeST, EquiGen-5) was from Bresagen Ltd. (Thebarton, Australia). Recombinant methionyl
194 bovine somatotropin (rMbST) was extracted from the slow release formula of Lactotropin®
195 syringes provided by Elanco (Greenfield, IN, USA) as described previously with
196 modifications [25]. Briefly, 20 mL of 50 mM CAPS buffer (pH 11.0; 100 mM NaCl) was
197 added to 500 mg of syringe content. The syringe content was emulsified by vortex for 1
198 minute and then by sonication for 10 minutes in a water bath. The emulsion was immediately
199 centrifuged (1000×g, 10 min) and the transparent layer containing rMbST was removed from
200 the white excipient layer. To ensure maximum recovery of rMbST a further 10 mL of CAPS
201 buffer was emulsified with the remaining white excipient and mixed by vortex and sonication
202 and centrifuged as previously described. The rMbST phases were pooled together and filtered

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203 using a 0.45 µm cellulose filter. The rMbST solution was immediately aliquoted, lyophilized
204 and stored at –20 °C until use. The protein concentration of the extracted rMbST was
205 determined using a BCA assay and confirmed by liquid chromatography coupled to high-
206 resolution mass spectrometry measurement (LC-HRMS^n; results not shown), as described
207 previously [11]. A tryptic digestion was performed overnight at 37 °C in 120 µL of 50 mM
208 ammonium bicarbonate, 10 mM EDTA and 1 µM pepstatin (pH 7.9) with 2 µg of enzyme.
209 The extracts were then evaporated, reconstituted in 40 µL of water/acetonitrile 70:30 (v/v)
210 with 0.2% (v/v) formic acid and analyzed by LC-HRMS^n. Separation of the peptides was
211 achieved on a Symmetry 300 2.1 mm × 150 mm C4 column packed with 3.5 µm beads, 300
212 Å pore size (Waters, Milford, USA) . The solvent flow rate was set at 300 µL min^{-1}. Peptides
213 were separated using acetonitrile containing 0.2% (v/v) formic acid (A) and water containing
214 0.2% (v/v) formic acid (B) as mobile phase. The elution gradient started with 10% A
215 increasing to 50% in 5 min, then decreasing to initial conditions in 5 min and remaining at
216 10% A for 5 more minutes. A divert valve was used to let the sample pass into the instrument
217 from 4.5 to 9 min. The typical expected retention time was 6.7 and 6.9 min for the rMbST *N*-
218 terminus peptide and the rMeST *N*-terminus peptide (used as internal standard), respectively.
219 The MS instrument was a linear ion trap coupled to an orbitrap allowing high resolution
220 measurements (LTQ-Orbitrap^{TM}, Thermo Electron, Bremen, Germany), fitted with an
221 electrospray ion source (ESI). The API interface was operated in positive ion mode. A sample
222 volume of 20 µL was loaded onto the column using the autosampler. A column heater was
223 used to ensure a stable column temperature of 30 °C. Mass spectrometric analyses were
224 performed in the following working conditions: capillary voltage was set at 42 V, source
225 voltage at 5 kV and capillary temperature at 300 °C. Nitrogen was used as sheath, auxiliary
226 and sweep gas at flow rates of 50, 10 and 10 (arbitrary unit), respectively. The linear ion trap
227 mass spectrometer was set to select the ions 933.5 and 913.3 corresponding to $[M+2H]^{2+}$ of

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228 the tryptic *N*-terminus peptides of rMeST and rMbST, respectively. Collision energy of 20%
229 (arbitrary unit) was applied to the ion 933.5 and 18% to the ion 913.3. The detection of the
230 resulting product ions was performed in the orbitrap at a resolution of 30 000. Acquisition
231 was performed in full scan mode from m/z 500 to 1500. Data were collected and analyzed
232 with the Xcalibur software (Thermo Electron).

233 **2.3. Preparation of immunogens**

234 *i) Coupling of rMbST to KLH*: the immunogen rMbST–KLH was obtained using the
235 EDC/NHS reaction. Briefly, 20 mg of EDC and 10 mg of NHS were dissolved in 100 µL of
236 0.05 M MES, 0.5 M NaCl buffer (pH 4.7). The solution of EDC/NHS was added to 10 mg of
237 rMbST and allowed to react for 10 min. The EDC/NHS-activated rMbST was added slowly
238 with stirring to 1 mL of PBS (pH 7.4) containing 20 mg of the carrier protein. The
239 conjugation reaction was performed overnight under stirring at room temperature.

240 *ii) Coupling of cysteine-containing rMbST N-terminus peptides to KLH*: synthetic
241 rMbST *N*-terminus-mimicking peptides, EP091213 and MF-MAP-C (Figure 1), were
242 conjugated to KLH via their cysteine residues using the commercial sulfo-SMCC conjugation
243 kit (Fisher Scientific UK, Leicestershire, UK) according to manufacture instructions.

244 All of the immunogens were purified by overnight dialysis (Slide-A-Lyzer Dialysis cassettes;
245 3kDa cut-off) against a 0.9% (w/v) NaCl solution. The protein concentration was estimated
246 by BCA assay and the conjugates were aliquoted, lyophilized and stored at −20 °C.

247 **2.4. Polyclonal antibody production**

248 Polyclonal antibodies were produced in New Zealand white SPF (specific pathogen free)
249 rabbits. A summary of the production of the pAbs is shown in Table 2. Rabbits were
250 immunized by subcutaneous injection with 1 mL of a 1:1 emulsion of a saline solution
251 containing the immunogen and Freund's complete adjuvant (first dose) or Freund's

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252 incomplete adjuvant (subsequent doses). Generally 0.2 mg of immunogen were administered,
253 with the exception of rMbST–KLH, for which 0.1 mg were used. Immunizations were
254 performed initially in intervals of 15 days, and then monthly after the third boost. A test
255 blood sample was obtained from every rabbit prior to immunization, and then 10 days after
256 each immunization from the 3rd boost onward. Pre-immune and immune sera were obtained
257 by coagulation and centrifugation of the blood samples. Working solutions were prepared in a
258 1:1 mixture of EIA buffer and ethylene glycol, and they were stored at –20 °C.

259 **2.5. Peptide-based affinity purification of the polyclonal antibody MU11**

260 The pAb MU11 was purified by affinity chromatography using a peptide EP093536 column.
261 *i)* *Peptide EP093536 affinity column preparation*: the $-NH_2$ groups of the Toyopearl
262 AF-Amino-650M gel were activated with MBS, followed by the coupling of the maleimide
263 activated $-NH_2$ moieties with the –SH group of the cysteine in the peptide EP093536. 2.5 mL
264 of gel in 1.25 mL of 50 mM sodium phosphate buffer (pH 6) were incubated for 30 min at
265 room temperature with orbital agitation with 175 µL of a MBS solution in *N*,*N*-
266 dimethylformamide (DMF; 15 mg/mL). The gel suspension was then centrifuged (1800×g, 2
267 min) and supernatant was discarded. The gel was washed 3 times with 2.5 mL of 10× PBS
268 (pH 7.4), and resuspended in 1.25 mL of PBS. Immediately prior to use, the peptide
269 EP093536 (8.4 mg) was dissolved in 1.4 mL of a mixture of DMF:PBS (30:70, v/v). The
270 peptide solution was added to the gel suspension and incubated for 18 h at room temperature
271 under orbital agitation. After centrifugation, the gel was resuspended in 2.5 mL of PBS and
272 packed into a purification column. The peptide EP093536 affinity column was stored at 4 °C.
273 *ii)* *Purification of the pAb MU11 by peptide-based affinity chromatography*: prior to the
274 peptide-based purification, the immunoglobulin fraction of the rabbit pAb MU11 was isolated
275 from the crude serum (5 mL) by protein A affinity chromatography. The recovered

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276 immunoglobulin fractions were then dialyzed in PBS (18 h, 4 °C), and concentrated in a
277 Centricon Plus-20 device (4600×g, 30 min, 4 °C) up to a final volume of 1 mL. The solution
278 containing the immunoglobulins was loaded onto the peptide EP093536 column pre-
279 conditioned by the addition of 5 volumes of PBS (20 g/L NaCl), and incubated at room
280 temperature for 3 h with orbital agitation. The non-retained immunoglobulins were eluted by
281 washing with PBS (20 g/L NaCl). The elution of the peptide-specific immunoglobulins was
282 conducted using 100 mM glycine buffer (pH 2.5). The column was regenerated by addition of
283 5 volumes of PBS and stored at 4 °C in 20:80 methanol:distilled water. Those fractions
284 containing immunoglobulins, as determined spectrophotometrically at 280 nm, were pooled
285 and dialyzed overnight in PBS containing 0.001% (w/v) NaN_3 at 4 °C. The antibody
286 solutions (specific and non-specific fractions) were concentrated as described above, and the
287 concentration was estimated by the BCA protein assay test. The antibodies were stored at –20
288 °C in PBS containing 0.001% (w/v) of thimerosal and 10% (w/v) of BSA.

289 **2.6. Antigen-coated competitive ELISA**

290 *i)* *General assay procedure:* ELISA plates were coated overnight at room temperature
291 with 100 µL of the standard solution [rMbST or bST] prepared in coating buffer (ranging
292 from 0.25 to 16 µg/mL). The blocking of the plates was performed by incubation for 2 h at 37
293 °C with 250 µL of blocking solution. After the coating and the saturation steps, the plates
294 were aspirated. In the competitive assay, 50 µL of the antibody solution and 50 µL of the
295 standards prepared in EIA buffer were added and incubated for 1 h at 37 °C. Then, 100 µL of
296 labeled secondary antibody prepared in EIA buffer (GAR–HRP at 1:2000) was added and
297 incubated for 1 h at 37 °C. Plates were washed 5 times with washing solution between each
298 incubation step. Finally, the retained peroxidase activity was revealed with 100 µL of a

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299 ready-to-use TMB solution for 30 min in the darkness at room temperature. The enzymatic
300 reaction was stopped by addition of 50 µL of 1.8 N H_2SO_4.

301 *ii)* *Optimized assay procedure:* the following procedure was used for the purified pAb
302 MU11. This protocol was based on the general assay procedure, with slight modifications. An
303 overnight pre-incubation of the antibody and the antigen (mixed at equal volumes in plastic
304 tubes) was performed at 4 °C. Then, 100 µL of the pre-incubated solutions were added to the
305 coated and blocked plates, and incubated for 30 min at 37 °C. A biotin/streptavidin
306 amplification system was used, consisting of an initial incubation with 100 µL of a 1:150000
307 dilution of GAR–b prepared in PBST (1 h, 37 °C), followed by an additional incubation with
308 100 µL of a 1:20000 dilution of STV–pHRP prepared in PBST containing 1% (w/v) of BSA
309 (1 h, 37 °C).

310 *iii)* *Signal processing:* Absorbance was monitored at 450 nm. The signal intensity was
311 plotted against the standard concentration in a logarithmic scale, and the resulting sigmoidal
312 curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot
313 software package from SPSS Inc. (Chicago, IL, USA). The IC_{50} value, corresponding to the
314 standard concentration that generates a 50% reduction of the maximum signal intensity
315 (A_{max}), was used for the estimation of the assay detectability. The limit of detection of the
316 assay (LOD) was determined as the concentration of standard that generates a 2sd decrease of
317 the signal obtained at the zero dose of analyte (A_0 – 2sd). The absorbance values were
318 normalized using A_0 as reference measure.

319 **2.7. Cross-reactivity study**

320 The capability of recognition by the peptide-purified antibody MU11 to pituitary bovine
321 somatotropin (bST), pituitary porcine somatotropin (pST), recombinant methionyl equine
322 somatotropin (rMeST), bovine serum albumin (BSA), lactoferrin, β-lactoglobulin, casein,

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323 bovine prolactin and bovine placental lactogen was evaluated by conducting competitive
324 experiments using the mentioned proteins as competitors. Cross-reactivity (CR) values were
325 calculated as follows: CR = $IC_{50(rMbST)}/IC_{50(competitor)} \times 100$.

326 **3. Results and discussion**

327 **3.1. Immunogen description**

328 In the present work several immunizing strategies for the production of anti-rMbST
329 antibodies were compared. The immunogens were categorized as i) complete recombinant
330 somatotropin (rMbST); ii) rMbST *N*-terminus-mimicking synthetic linear peptide; and iii)
331 rMbST *N*-terminus-mimicking synthetic MAP. In the case of the synthetic molecules (linear
332 peptide and MAP), the design comprised a *C*-terminus cysteine, an amino acid commonly
333 introduced for coupling purposes, via the side chain thiol group. The rMbST-mimicking
334 synthetic linear peptide EP091213 represents the first 17 *N*-terminus amino acids of rMbST
335 (H_2N–MFPAMSLSGLFANAVLRC–COOH). The rMbST-mimicking synthetic multiple
336 antigen peptide MF-MAP-C (Figure 1), displays in an *arachnid*-type manner eight units of
337 the first two *N*-terminus amino acids of rMbST, methionine and phenylalanine, with a weakly
338 immunogenic $lysine_n$ core. All of the immunogens were prepared by covalent conjugation of
339 the three mentioned molecules to the carrier protein KLH. In order to guarantee the
340 accessibility of the rMbST *N*-terminus characteristic portion of the antigens to the immune
341 system, different coupling chemistries were used for the preparation of the immunogens. In
342 the case of the recombinant whole protein, formation of amide bonds between the free
343 carboxylic groups of rMbST and the amine groups of KLH was conducted using the active
344 ester method. The peptide EP091213 and MF-MAP-C were conjugated using the

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345 heterobifunctional crosslinker sulfo-SMCC, by coupling each peptide to maleimide-activated
346 amine groups of KLH via the cysteine residue of their *C*-terminus.
347 By the immunization of rabbits with the three mentioned immunogens, 9 polyclonal
348 antibodies were obtained (Table 2).

349 **3.2. Preliminary antibody characterization**

350 The capability of immunodiscrimination of the 9 available pAbs between the native and the
351 recombinant methionyl somatotropins was initially assessed by a checkerboard titration
352 procedure in the antigen-coated ELISA format using both proteins as coating antigens. As a
353 first approach, the crude sera were analyzed without any further purification. As shown in
354 Table 3, all three immunogens gave rise to a positive immune response. All of the antibodies,
355 with only one exception (M10), recognized the native somatotropin and/or the recombinant
356 methionyl isoform to a different extent. Despite immunization with rMbST–KLH generated
357 the pAbs with the highest titers, these were unable to immunodiscriminate the recombinant
358 methionyl isoform, therefore displaying a very similar binding behavior towards both
359 somatotropins. From these results and those described in previous studies where the same
360 immunizing strategy was used [21, 23-25], it could be inferred that using the complete
361 recombinant protein as immunogen directs the immune response towards common antigenic
362 determinants within the recombinant and the native isoforms, with the rMbST *N*-terminus
363 being "masked". This remark is of special relevance in the case of pAbs, representing a
364 heterogeneous collection of antibodies with disparate profiles of selectivity, where the
365 rMbST-specific sub-population, if produced, would consequently be in the minority. Finally,
366 the immunogens consisting of rMbST *N*-terminus-mimicking synthetic peptides (both the
367 linear peptide and the MAP), afforded the most promising results, as three of the produced
368 pAbs exhibited a higher recognition towards rMbST, namely M9 for EP091213–KLH, and

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369 MU9 and MU11 for MF-MAP-SMCC–KLH. These antibodies were therefore selected for
370 further studies.
371 In order to reduce background-associated problems encountered with the raw sera, the three
372 pAbs were purified by protein A affinity chromatography. A representative set of data of the
373 response to rMbST and bST of the mentioned protein A purified antibodies by checker-board
374 titration in the antigen-coated ELISA is displayed in Figure 2, including also one of the
375 generic pAbs (BC8) for comparative purposes. The highest rMbST/bST recognition ratio was
376 observed for pAb MU11, therefore highlighting the superior efficiency of the immunogen
377 based on the rMbST *N*-terminus-mimicking MAP coupled to KLH over the other strategies
378 used. Despite the fact that suitable amino acid number in MAPs is usually considered to be
379 comprised between 10 and 20 residues [35], the immunization with an rMbST *N*-terminus
380 dipeptide-mimicking MAP has been proven to be sufficiently immunogenic to induce the
381 production of anti-rMbST antibodies.
382 In conclusion, as previously reported by other authors regarding the production of antibodies
383 directed to other targets [33, 36], the immunization with a MAP resulted in a stronger and/or
384 more specific response than that generated by a mimicking monovalent peptide or the
385 complete recombinant protein. According to the results herein presented, the rabbit pAb
386 MU11 was chosen for the development of an anti-rMbST immunoassay.

387 **3.3. Competitive immunoassay based on the polyclonal antibody MU11**

388 Those combinations of antibody dilution/coating antigen (rMbST) affording adequate signal
389 intensity (around 1 absorbance units), as determined by checker-board titration, were selected
390 to perform inhibition experiments using rMbST and bST as competitors. A preliminary
391 experiment was carried out to determine the assay conditions generating the highest
392 inhibition ratios for rMbST. It was found that the introduction of a pre-incubation step of the

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393 antibody and the competitor in combination with a short period of time for the competitive
394 step provided an improvement of the assay sensitivity (results not shown). Compensation of
395 signal loss due to the reduction of the immunoreactive step time was achieved using a signal
396 amplification system based on a biotinylated secondary antibody and HRP–labeled
397 streptavidin. As shown in Figure 3, a specific response was displayed by the antibody MU11
398 towards rMbST, whereas no inhibition was observed when bST was used as competitor. All
399 of the evaluated combinations afforded very similar inhibition ratios for rMbST, with an
400 estimated IC_{50} value comprised between 500 and 5000 µg L^{-1}. In order to improve the
401 antibody performance in terms of sensitivity, the pAb was subjected to a further purification
402 step using an rMbST *N*-terminus-mimicking synthetic peptide-based affinity procedure. The
403 affinity purification of antibodies produced against rMbST has been previously reported,
404 although in all of the described methods the complete recombinant protein was used with this
405 purpose [16, 17, 21, 25]. The competitive standard curves for rMbST and bST, as well as the
406 assay conditions and parameters obtained using the peptide-purified MU11 pAb in the most
407 sensitive coating antigen/antibody dilution combination are included in Figure 4. By using
408 the peptide-purified fraction of the pAb MU11 the assay sensitivity was greatly improved,
409 being the resulting LOD for rMbST in buffer of 66 µg L^{-1}. This result indicates that antigen-
410 based affinity purification is a convenient strategy not only to isolate the immunoglobulin
411 sub-populations directed to a ligand, but also to modulate the overall avidity of a pAb, and
412 thus the assay sensitivity, as those immunoglobulins with the lowest affinity towards the
413 target are removed during the washing step. Levels of rMbST/bST in fresh bovine milk after
414 administration of somatotropin slow release formulations have been reported to be below 5
415 µg L^{-1} [15, 37], in contrast to plasma or serum, where concentrations up to 120 µg L^{-1} have
416 been found [13]. Furthermore, common heat treatments to which commercial milk is
417 submitted prior to commercialization, such as pasteurization, reduce up to 90% the

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418 rMbST/bST content [14, 37]. In order to effectively monitor the presence of rMbST in milk
419 samples, further improvement of the MU11-based immunoassay herein presented is therefore
420 required, or alternatively, the production of additional receptors which display the specificity
421 to rMbST shown by pAb MU11, altogether with an increased affinity towards the target. As a
422 first approach, immunization with rMbST-mimicking MAPs bearing longer peptides will be
423 attempted, in order to determine the influence of the length of the displayed subunits in the
424 MAP on the avidity of the produced antibodies.

425 **3.4. Cross-reactivity analysis**

426 The interaction of the peptide-purified MU11 pAb with bST and somatotropins from other
427 species (either native or recombinant; pST and rMeST), BSA, milk bovine proteins
428 (lactoferrin, β-lactoglobulin and casein), as well as bovine proteins displaying a high
429 sequence homology with bST (lactogen and prolactin) was assessed in a cross-reactivity
430 study, using the mentioned molecules as competitors. No recognition was observed to any of
431 the evaluated proteins, with the exception of rMeST, for which a CR value of 5.6% was
432 obtained. The pituitary somatotropins which were not recognized by MU11, bST and pST,
433 lack the *N*-terminus methionine present in the dipeptide displayed by the immunogen used for
434 the production of the antibody (MF-MAP-SMCC–KLH), finding which points out the crucial
435 role of this residue as antigenic determinant in the antibody-antigen binding event.
436 Comparing the *N*-terminus amino acid sequence of rMbST (H_2N–
437 MFPAM**S**LS**G**LFANAVLRA–) with that of rMeST (H_2N–MFPAM**P**LS**S**LFANAVLRA–),
438 whereas both recombinant proteins present the *N*-terminus methionine, two differences are
439 encountered (highlighted in bold). Given the decreased interaction observed for rMeST with
440 respect to rMbST, serine at position 6 and/or glycine at position 9 appear to be also required
441 for the antibody recognition. Presumably, the additional purification of the antibody using the

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442 rMbST *N*-terminus-mimicking linear peptide, would have contributed to the selection of
443 immunoglobulins which paratope would better fit an epitope containing these two amino
444 acids, therefore being specific for rMbST.

445 **4. Conclusions**

446 For decades the production of specific antibodies directed towards rMbST has not been
447 successfully accomplished as a consequence of an extremely minor difference (one amino
448 acid) encountered at the *N*-terminus of both proteins. In this paper, we report the generation
449 of a rabbit pAb displaying a high selectivity towards rMbST in a competitive antigen-coated
450 ELISA format. The immunogen employed for the production of the pAb consisted in an
451 rMbST *N*-terminus-mimicking synthetic MAP displaying the first two amino acids of rMbST
452 conjugated to the carrier protein KLH. Further purification of the antibody using an rMbST
453 *N*-terminus-mimicking synthetic linear peptide significantly improved the performance of the
454 antibody. Further work is currently ongoing in order to produce rMbST-specific monoclonal
455 and polyclonal antibodies to efficiently develop an immunoassay that meets the requirements
456 both in terms of specificity and sensitivity to be implemented for the routine screening of
457 rMbST in milk.

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529 **Table and Figure captions**

530 **Table 1**. Pituitary and commercial recombinant bST specifications.

531 **Table 2**. Polyclonal antibody production.

532 **Table 3**. Summary of the preliminary characterization of the pAbs by antigen-coated ELISA.

533 **Figure 1**. Chemical structure of the rMbST *N*-terminus-mimicking immunizing synthetic
534 multiple antigen peptide (MAP).

535 **Figure 2**. Recognition towards rMbST and bST coating antigens (4 µg mL^{-1}) displayed by
536 different pAbs.

537 **Figure 3**. Competitive experiment performed with the protein A purified pAb MU11 using
538 bST and rMbST as competitor reagents (empty and filled bars, respectively). rMbST coating
539 antigen concentrations were 1 (■), 2 (■), 4 (■), 8 (■) and 16 (■) µg/mL, and they were
540 combined with the following antibody dilutions: 1/500, 1/500, 1/1000, 1/1500 and 1/2000,
541 respectively. Each value represents the average of three independent experiments.

542 **Figure 4**. Standard curves for rMbST and bST obtained with the peptide-purified pAb
543 MU11.

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545 **Highlights**

546

547

548 - Production of polyclonal antibodies directed to recombinant methionyl bovine
549 somatotropin (rMbST)
550 - Multiple antigen peptide mimicking rMbST *N*-terminus used as immunogen
551 - Immunodiscrimination between native and recombinant bovine somatotropins by
552 ELISA

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Table 1. Pituitary and commercial recombinant bST specifications

Somatotropin	aa length	Molecular weight	Position 1	Position 1/2	Position 126/127
bST variant 1	191	21788 Da	Ala-[c]	-Phe-[d]	-Val-[e]
bST variant 2[a]	191	21802 Da	Ala-	-Phe-	-Leu-[f]
bST variant 3	190	21717 Da		Phe-	-Val-
bST variant 4	190	21731 Da		Phe-	-Leu-
rMbST (Posilac®)[b]	191	21851 Da	Met-[g]	-Phe-	-Leu-

[a] Majority isoform of pituitary bST. [b] Commercial recombinant bST most commonly used worldwide. [c] Alanine. [d] Phenylalanine. [e] Valine. [f] Leucine. [g] Methionine.

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Table 2. Polyclonal antibody production

Immunogen	Coupling chemistry	pAbs
rMbST–KLH[a]	active ester method (–CO_2H groups in rMbST; –NH_2 groups in KLH)	BC5, BC6, BC7, BC8
EP091213–KLH[b]	sulfo-SMCC method (–NH_2 groups	M9, M10
MF-MAP-SMCC–KLH[c]	in KLH; –SH group of Cys[d] in peptides)	MU9, MU10[e], MU11, MU12

[a] Complete recombinant methionyl bST. [b] Synthetic rMbST *N*-terminus-mimicking linear peptide. [c] Synthetic rMbST *N*-terminus-mimicking multiple antigen peptide. [d] Cysteine. [e] This rabbit died during the immunization process.

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Table 3. Summary of the preliminary characterization of the pAbs by antigen-coated ELISA

Immunogen	pAb	Binding to rMbST	Binding to bST
rMbST–KLH	BC5	+++[a]	+++
	BC6	+++	+++
	BC7	+++	+++
	BC8	+++	+++
EP091213–KLH	**M9**	+	+
	M10	–	–
MF-MAP-SMCC–KLH	**MU9**	+	+
	MU11	+	–
	MU12	+++	+++

[a] Results corresponding to the analysis of the raw pAbs used without any further purification. Binding to coating antigens (rMbST and bST at 1 µg mL^{-1}) corresponding to a 1/500 dilution of the pAbs expressed as: (+++) strong (AU ≥ 2); (++) medium (1 ≤ AU < 2); (+) low (0.3 ≤ AU < 1); (–) negligible (AU < 0.3). Those pAbs providing a signal intensity for rMbST at least 0.2 UA above that observed towards bST have been highlighted in bold.

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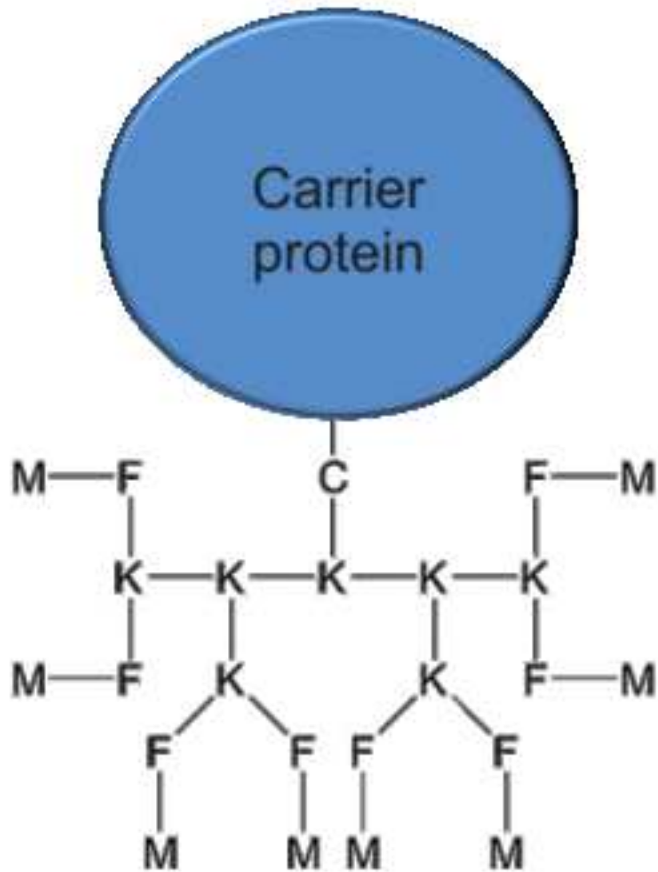
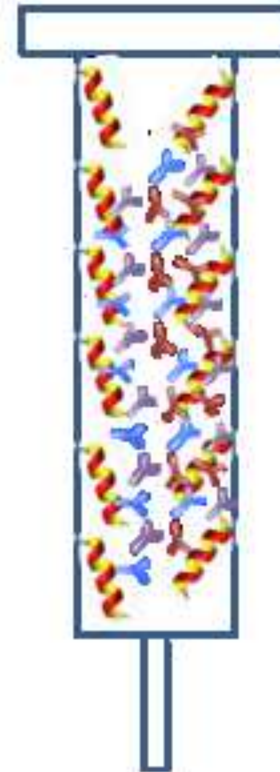
*Graphical Abstract

Immunodiscrimination between closely structurally related proteins

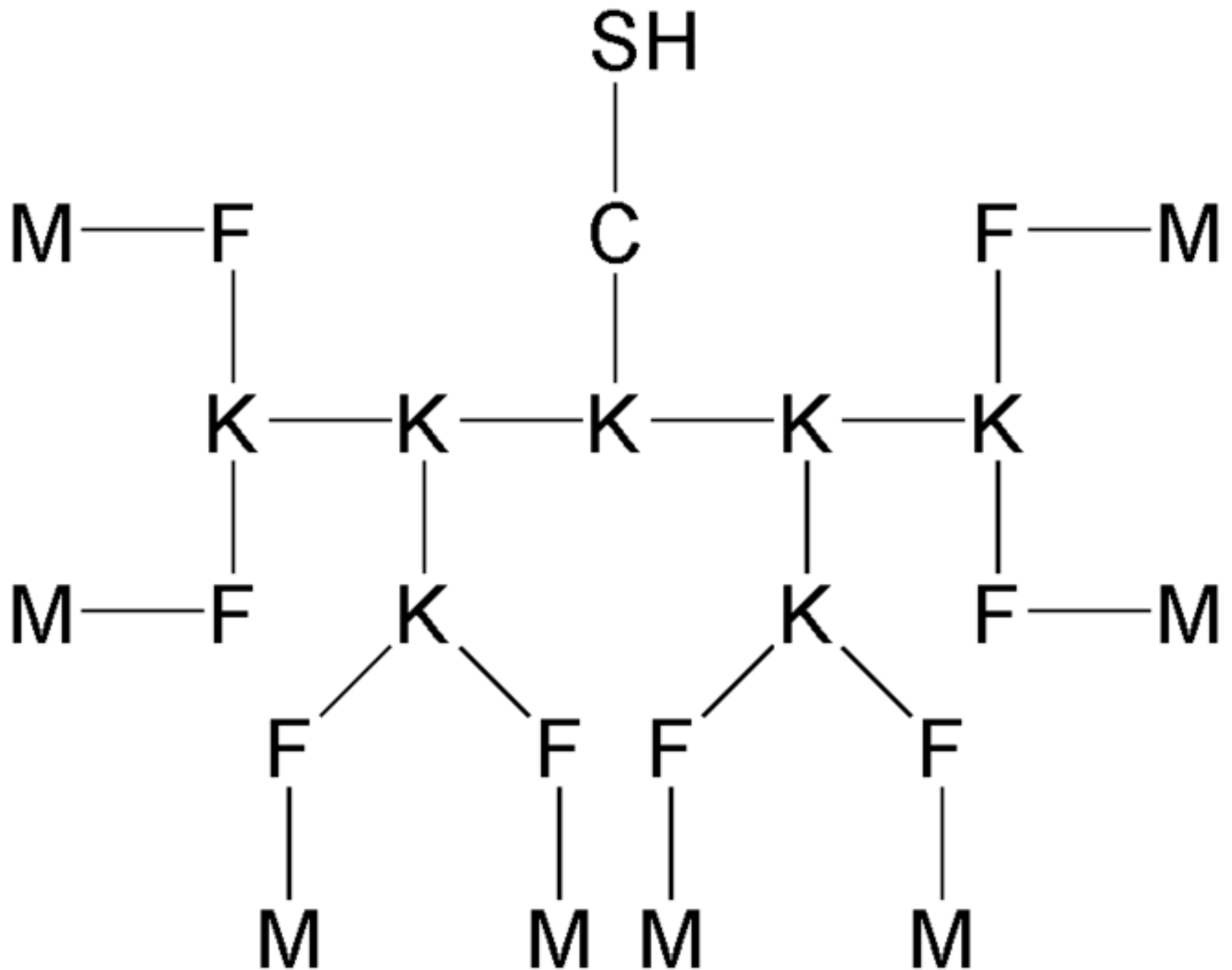




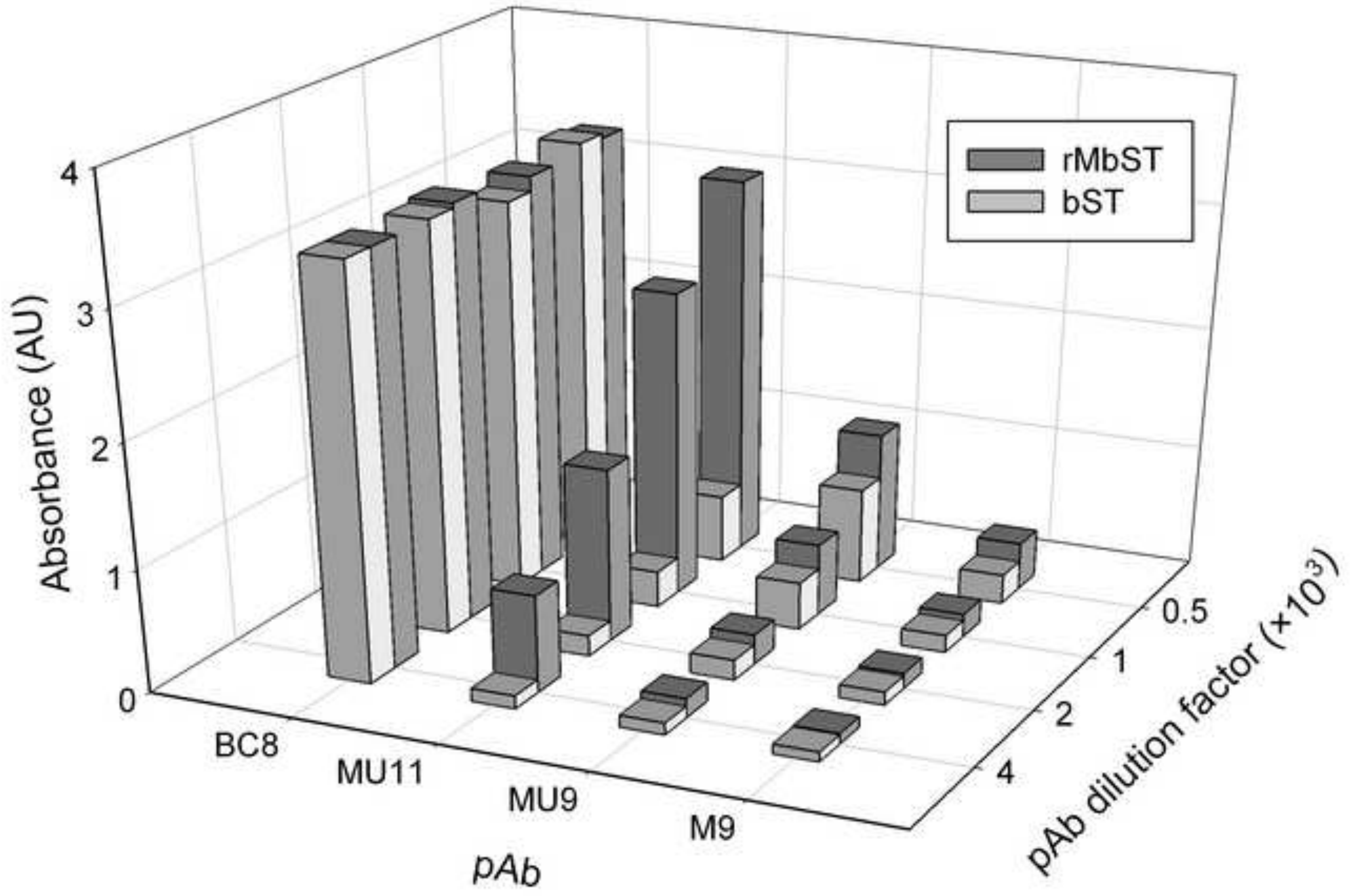
MAP-based immunogen

Monovalent peptide-based affinity purification of antibodies

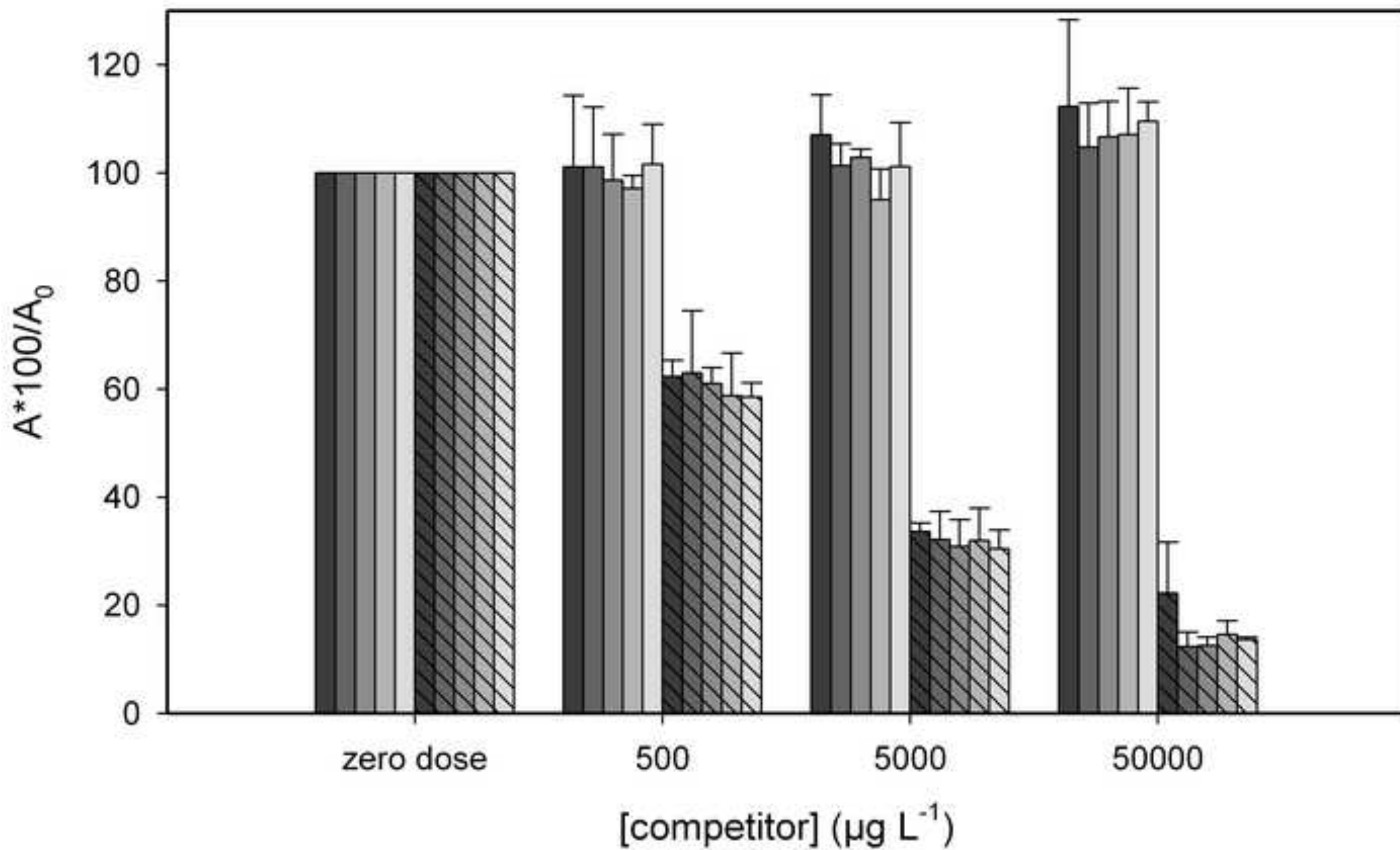
Figure



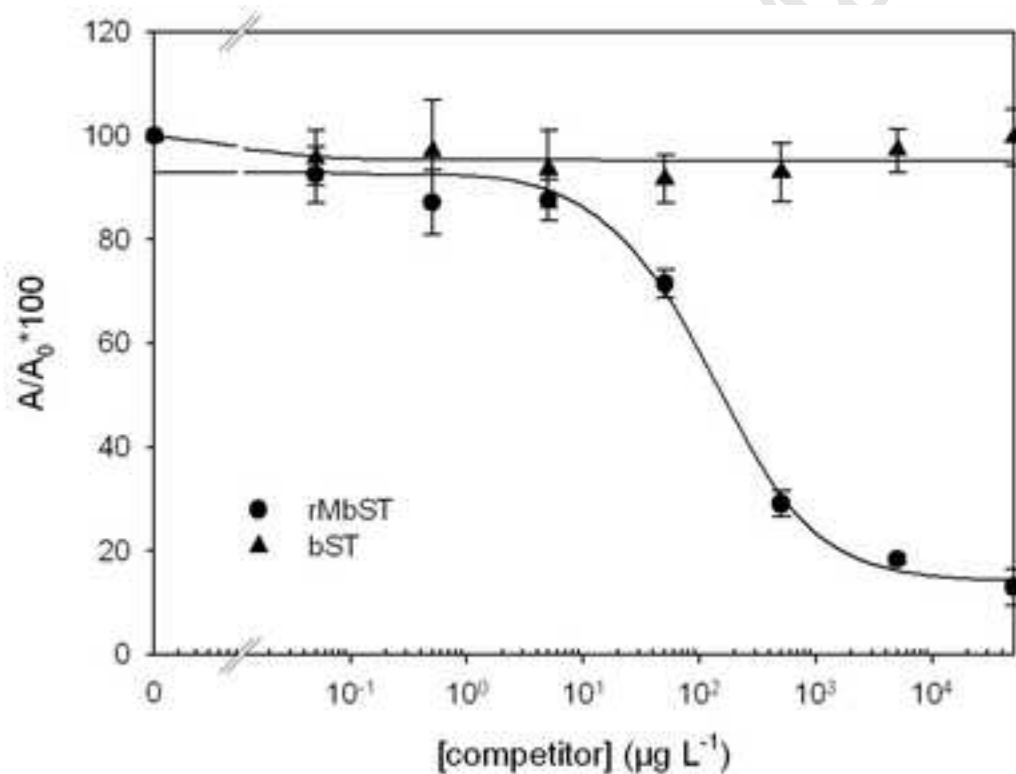
Figure



Figure



Figure



Assay conditions and analytical parameters of the rMbST standard curve

antibody dilution	1/2000
coating (rMbST)	2 µg mL^{-1}
pre-incubation antibody + competitor	overnight (4 °C)
competition	30 minutes (37 °C)
A_{max} [a]	1.01 ± 0.18
slope	−0.96 ± 0.20
IC_{50} (µg L^{-1})	128 ± 16

[a] Each value represent the average ± sd of 4 independent experiments .