DETECTION OF ILLEGAL GROWTH PROMOTERS IN MEAT-PRODUCING ANIMALS : DEVELOPMENT OF RECEPTOR ASSAYS USING RECOMBINANT HORMONE-BINDING DOMAINS OF HUMAN STEROID RECEPTORS

M.-L. Scippo*, M. Lion**, E. Baise**, J.-M. François**, T. Corbaye*, A. Duyckaerts*, F. Rentier-Delrue**, M. Muller**, G. Maghuin-Rogister* and J.A. Martial**

* Laboratory of Analysis of Foodstuffs of Animal Origin, Faculty of Veterinary Medicine, University of Liège, B-43 bis, Sart Tilman, 400 LIEGE, BELGIUM
** Laboratory of Molecular Biology and Genetic Engeneering, Faculty of Sciences, University of Liège, B6, Sart Tilman, 4000 LIEGE, BELGIUM

Abstract

In this study, we are setting up receptor assays allowing the detection of residues with estrogen, progestagen, androgen or corticosteroid activities by using recombinant proteins corresponding to the hormone-binding domain of the respective receptors.
We show here results obtained with the estrogen receptor. Its hormone-binding domain was fused in frame to the C-terminus of the *S. japonicum* glutathion-S-transferase (GST) coding region and expressed in *E.coli*. Expression conditions were worked out to optimise the production of soluble protein. In combination with the overexpression of *E.coli* chaperones GroEL and GroES, we were able to produce up to 40 mg of soluble estrogen receptor hormone-binding domain per litre of *E.coli* culture, with specific hormone binding activity as evidenced by radioreceptor assay (RRA) studies.
The proposed RRA allows the detection of concentrations of 0.8 ppb of 17β estradiol in urine. The soluble recombinant receptor is stable at least for 6 months in several storage conditions (4°C, -20°C, -70°C or lyophilised).

Introduction

The control of the use of growth promoters in animal production has become an important issue. Illegal treatments are applied using various substances : steroid derivatives with sex hormone activities (androgen, estrogen or progestagen), glucocorticoids, or β-adrenergic agonists. Generally, the molecular structure of the anabolizing agent is not known. Thus, when screening methods like immunoassays are used for the control, their high specificity generally limits the number of controlled substances to 5 or 6. Compared to the number of different substances (more than 50) that have been identified in cocktails or in injection sites, it is clear that another screening strategy is needed.

Detection of all target compounds with a single multi-residue method is possible when using receptor assays. Receptor assays have already been developed to detect estrogen hormones (1) or β-adrenergic agonists (2), by using receptors extracted from bovine tissues. In order to

avoid the problems due to inhomogeneity and instability of such preparations from tissues, we are setting up receptor assays using recombinant receptors produced in *E.coli*.

In this paper, we describe the production of a recombinant estrogen receptor and its use in the developmnent of a radioreceptor assay to detect estrogen hormones in urine samples.

Materials and Methods

Recombinant receptor expression

Expression vector. Sequences coding for the hinge region and the hormone binding domain of the human estrogen receptor (ER) (figure 1) were fused in frame to the C-terminus of the *S.japonicum* glutathion-S-transferase gene (GST), present in the pGEX-4T-3 bacterial expression plasmid (Pharmacia). The resulting plasmid pGEX-ER was transformed into the *E.coli* strain BL21 (ompT-, lon-), for expression of the GST-ER fusion protein following induction with isopropyl-β-D-thiogalactoside (IPTG). To facilitate the folding of the GST-ER protein, expression vectors encoding the GroEL and GroES chaperone proteins (3) were co-transformed.

Induction and lysis protocol. *E.coli* BL21 harbouring pGEX-ER and pGRoEL/S were grown overnight in 50 ml of Luria-Bertani medium (LB) containing 100 µg ampicillin/ml and 30 µg chloramphenicol/ml. The culture as then transferred to 1 litre of fresh LB with both antibiotics. When the culture has reached an OD_{600} of 0.9, synthesis was induced with 1 mM IPTG and culture was grown for an additional 15h at 18°C. After centrifugation, bacterial pellets were resuspended and the bacteria were broken in a French cell press at 10^8 Pa. After centrifugation for 2 h at 30,000 g, the pellet and the supernatant were analysed by SDS-PAGE and Western blot using rabbit antibodies raised against a peptide corresponding to the C-terminal aminoacids 576-595 of the human ER, and peroxidase conjugated secondary antibody against rabbit IgG.

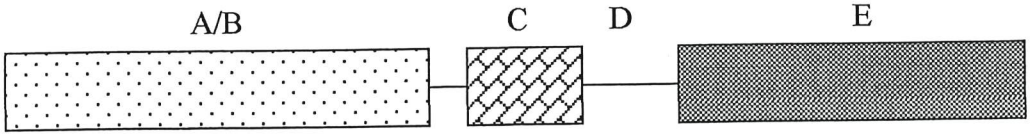

Figure 1 : Structure of the human intracellular estrogen receptor (4). A/B : Modulation domain; C : DNA binding domain; D: Hinge region; E: hormone binding domain.

Radioreceptor assay (RRA)

Receptors (30,000 g supernatant after *E.coli* expression) were incubated for 2 h at 25 °C with ^{3}H-17ß-estradiol (5 nM) and steroid standard solutions (or sample extracts). Non specific binding was determined by adding an excess of estradiol standard. Bound and free fractions were separated by addition of dextran-coated charcoal suspension and centrifugation.

To evaluate the receptor amount, Scatchard analysis was performed using this protocol.

Results

High expression of recombinant estrogen receptor in *E.coli*

Co-expression of GST-ER with *E.coli* chaperones GRO-EL and Gro-ES resulted in the production of up to 40 mg of soluble fusion protein per litre of *E.coli* cells (figure 2A), which is recognized by the specific ER antibody (figure 2B). A low amount of insoluble immunoreactive material was produced as well (figure 2B).

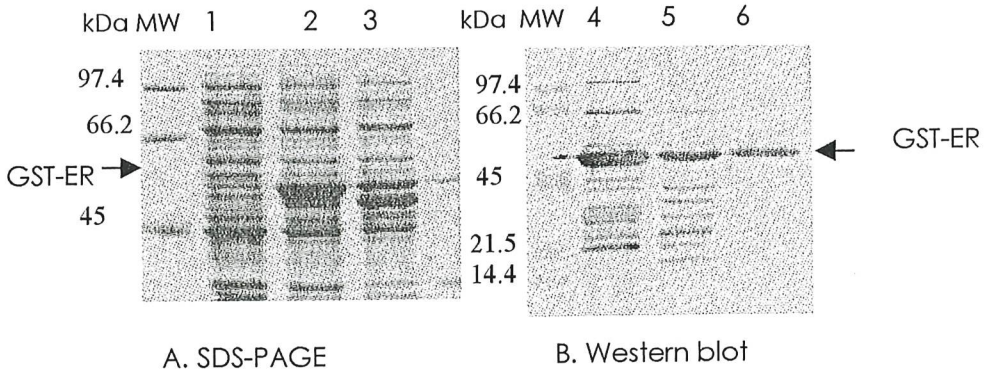

Figure 2 : A. SDS-polyacrylamide gel of E.coli BL21 proteins without (1) or with (2) IPTG induction. 3 : supernatant of the 30,000 g centrifugation.
B. Western blot analysis of IPTG-induced E.coli BL21 proteins (4. Supernatant (5) and pellet (6) of the 30,000 g centrifugation.

Binding study

A crude preparation of the GST-ER fusion protein was tested for its affinity to 17β-estradiol . Scatchard analysis (figure 3) revealed a K_D of 0.5 10^{-9} M, consistent with the affinity of the native receptor .

The concentration of estrogen binding molecules in the assay allows us to conclude that more 95% of the soluble bacterial expressed GST-ER fusion proteins are able to bind hormone.

The relative binding activity (RBA) of various estrogens was assessed by measuring the binding inhibition of ^{3}H-17β-estradiol to the GST-ER fusion protein caused by increasing concentrations of various estrogenic substances and other steroids (figure 4).

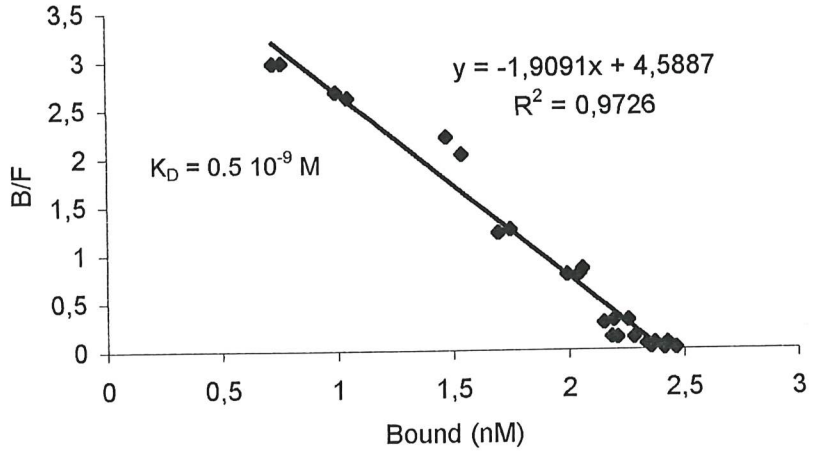

Figure 3 : Scatchard analysis of the crude preparation of GST-ER.

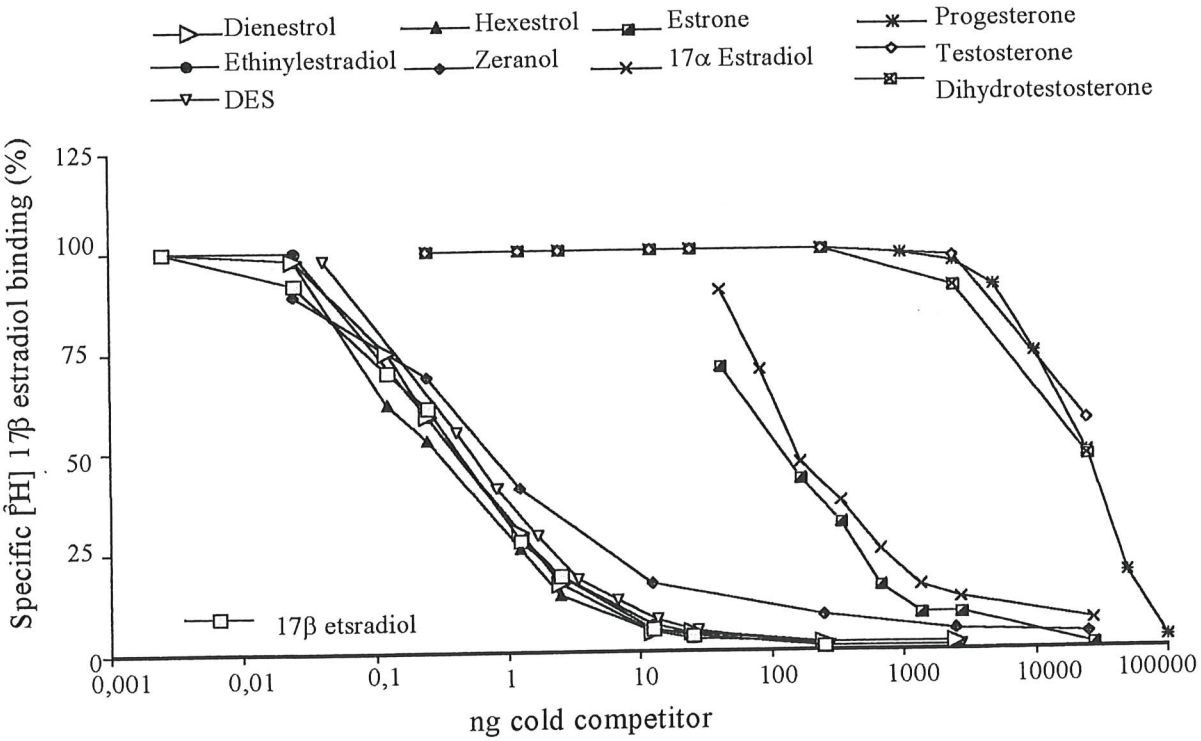

Figure 4 : Crude extracts of E.coli expressing the estrogen receptor fusion protein GST-ER were incubated with 5 nM [^{3}H] 17β-estradiol and varying concentrations of competitor steroid as indicated.

The crude bacterial extract containing the soluble fusion protein GST-ER was stored in various conditions (4°C, -20°C, -70°C or lyophilised) during 6 months without loss of ligand binding capacity (data not shown).

RRA for the detection of estrogen hormones in urine

The detection limit measured in bovine urine goes down to 0.8 ppb for 17β-estradiol (E2). Blank urine samples were spiked with 17β-estradiol. The resulted obtained (Table 1.A) are in the range of the acceptable variations defined in the 93/256 European doucment (5).
We determined estrogen concentrations (in terms of "apparent" E2 concentration) in HPLC fractions prepared from bovine urine samples collected as part of the official supervision program for illegal use of anabolics in the Netherlands (Dr C. Arts, TNO, Zeist, The Netherlands) (Table 1B). Measured concentrations are consistent with those obtained at the TNO.

A. Spiked urine samples with 17ßE2		**B. Real urine samples***	
Added	Found	1) negative samples : < L.D.	
1 ppb	1.3 ± 0.5 ppb (n = 7)	2) positive samples (n=3)	3) spiked samples* (5ppb) (n=3)
2 ppb	2.6 ± 0.8 ppb (n=7)	1.3 ± 0.3 ppb	5.9 ± 0.5 ppb
5 ppb	6 ± 0.5 ppb (n=5)	1.9 ± 0.6 ppb	5.3 ± 0.6 ppb
		2.6 ± 0.5 ppb	4.5 ± 0.4 ppb
		8.5 ± 1.9 ppb	4.8 ± 0.3 ppb
		4.8 ± 0.8 ppb	

Table 1 : Results of RRA analysis of bovine urine samples (: HPLC fractions).*

Conclusions

In conclusion, this study shows that the use of recombinant human steroid receptor hormone-binding domains can be helpful for the development of a multianalyte screening method, in various fields. These fields are for example the control of hormones in meat products, the

anti-doping control in human athletes as well as in racing/sport horses or dogs, or screening of endocrine disruptors in environmental samples.

References

1. Arts, C.J.M., Kemperman, P.T.W., Van Den berg, H. 1989. Estrogen radioreceptor assay for multi-residue screening of bovine urine for estrogenic anabolic compounds. Food Add. Contam., **6**, p.103 – 106.
2. Helbo, V., Vandenbroeck, M., Maghuin-Rogister, G. 1994. Development of a radioreceptor assay for β adrenergic agonists. Arch. Lebensmittelhyg., **45**, p.57-61.
3. Goloubinoff, P., Gatenby, A.A., Lorimer, G.H. 1989. GroE heat-shock proteins promote assembly of foreign prokariotic ribulose biphosphate carboxylase oligomers in *Escherichia coli*. Nature, **337**, p. 44-47.
4. Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bonert, J.-M. and Chambon, P. 1986. The chicken estrogen receptor sequence : homology with v-erbA and the human estrogen and glucocorticoid receptors. Embo J., **5**, p. 891-897.
5. Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or a thyrostatic action (O.J. N° L 118 of 14.5.93, p. 64).

Aknowledgements

We thank the Ministry of the Walloon Region (DGTRE) and the Federal Ministry of Agriculture (DG6) for their financial support.

We thank Dr C. Arts (TNO, Zeist, NL) for the gift of HPLC fractions prepared from bovine urine samples.