HOMOGENEOUS AND HETEROGENEOUS ENZYMEIMMUNOASSAYS FOR THE HOME DETECTION OF FERTILITY

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ABSTRACT

The physiology of the menstrual cycle has been reviewed and the suitability of the ovarian steroid urinary metabolites estrone glucuronide and pregnanediol glucuronide as markers of fertility and as utilised by the home Ovarian Monitor fertility assay was discussed. The biomaterials for the homogeneous enzymeimmunoassay which forms the basis of the Ovarian Monitor home fertility assay were prepared. One of the major difficulties in preparing a signal generator for use in home tests is the separation of the unconjugated enzyme material from the desired signal generators. In this thesis a new procedure was developed for the purification and isolation of the complex range of signal generators formed during acylation of hen egg white lysozyme with estrone glucuronide. A cation exchange column in the presence of 7 M urea allowed the separation to be carried out in the absence of the hydrophobic effects which complicate other schemes. Even under these conditions complex behaviour was seen which could be rationalised in terms of the tertiary structures of the conjugates and their electrostatic fields. A second step involving hydrophobic interaction chromatography gave two pure mono conjugated estrone glucuronide lysozyme conjugates in good yield the activities of which were highly inhibited (>90%) by anti-estrone glucuronide antibodies. The availability of the pure conjugates allowed the effect of tertiary structure on the immunoassays to be evaluated. The results showed that both mono acylated hen egg white lysozymes could be used to give good standard curves for use in monitoring menstrual cycles for the naturally occurring periods of fertility and infertility. Since the specific activity of human lysozyme is three times that of hen egg white lysozyme, in an attempt to provide a more rapid test for fertility human lysozyme estrone glucuronide conjugates were synthesised with estrone glucuronide for the first time. However, despite the fact that these two enzymes had very similar tertiary structures they behaved completely differently in the protein chemistry and immunological experiments reported in this thesis. The human enzyme was more easily acylated to give pure mono acylated conjugates in high yield and the conjugates were more easily purified to give highly inhibitable conjugates (>95%). The differences in behaviour could be accounted for in terms of the sequence differences between the two lysozymes and the relative exposure of the lysine residues. A fast assay (1-2 minutes) was developed for urinary estrone glucuronide using the three new signal generators. However, the sensitivity of the assays was less than half that of the hen egg white conjugates making them unsuitable for use in home assays for fertility. The assays could be useful for women using fertility drugs such as clomiphene. The lack of sensitivity of the assays and other binding behaviour indicated a much tighter binding to the antibody than with the hen egg white conjugates. This important difference was accountable on the basis of the extra extension of the lysine residues in the human enzyme. A new method for producing estrone glucuronide conjugates of the active enzyme horse radish peroxidase was evaluated. The mono substituted hemin conjugates reconstituted with the apo protein to give active peroxidases with good specific activities (~50%) and good stability. The procedure is such that any small molecule can be attached to the enzyme using the same procedures and a large range of signal generators can be formed for immunoassays. However, both assay formats examined failed to produce an assay in this thesis. The reconstituted enzymes, although binding to the immobilised anti- estrone glucuronide antibodies as required did not produce the necessary colours. The reasons for this were discussed.

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ABBREVIATIONS

ΔT	change in transmission
A_{278}	absorbance at 278 nm
A_{404}	absorbance at 404 nm
Ab	antibody
Abs	absorbance
Ag	antigen
AS	antiserum
BBT	basal body temperature
BSA	bovine serum albumin
C_b	conjugate bound
C_t	total conjugate
Cj	conjugate
CM	carboxymethyl cellulose
CV	co-efficient of variation
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DE	diethylaminoethyl resin
ELISA	enzyme linked immunosorbent assay
E1-E10	hen egg white lysozyme conjugates labelled according to elution order under standard conditions on the Mono-S column with 7 M urea buffers
E1G	estrone glucuronide (refers to acid form)
E1G-(H)	estrone glucuronide acid form
E1G-(Na)	estrone glucuronide sodium salt
eq	equilibrium
EC_{50}	analyte mid-point of a normalised standard curve
ESMS	electrospray mass spectroscopy
FAD	flavin adenine dinucleotide
FPLC	fast protein liquid chromatography
FSH	follicle stimulating hormone
HCG	human chorionic gonadotrophin
HE1-HE5	human lysozyme conjugates labelled according to elution order under standard conditions on the Mono-S column with 7 M urea buffers
HEWL	hen egg white lysozyme
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HuL	human lysozyme
i.d.	internal diameter
IgG	immunoglobulin type G
IR	initial rate
K_a	equilibrium association constant
k_{cat}	enzyme turnover number
K_d	equilibrium dissociation constant
K_{d1}	equilibrium dissociation constant for E1G with anti-E1G antibody

K_{d2}	equilibrium dissociation constant for E1G-conjugate with anti-E1G antibody
K_m	Michaelis Mentis constant - measure of substrate affinity
k_{off}	dissociation rate constant
k_{on}	association rate constant
L	lysozyme
LAM	lactational amenorrhoea method
LH	luteinising hormone
MEK	methyl ethyl ketone (2-butanone)
M. lysodeikticus	*Micrococcus lysodeikticus* (bacteria particularly susceptible to lysis by lysozyme
Mr	molecular weight
M.W	molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NFP	natural family planning
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PEG	polyethyleneglycol
PdG	pregnanediol glucuronide
PGLIA	prosthetic group labelled immunoassay
pI	isoelectric point
r	correlation co-efficient
RIA	radioimmunoassay
RZ	Reinheits zahl (purity number)
SA	specific activity
std	standard
T	transmission
TLC	Thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
V_{max}	maximal enzymatic rate
WHO	World Health Organisation

H	hemin
HA	hemin-aminohexanoic acid mono-conjugate
HA(di)	hemin-aminohexanoic acid di-conjugate
HAME	hemin-aminohexanoate methyl ester mono-conjugate
HH	hemin-hexylamine mono-conjugate
HLysE1G	hemin-lysine-estrone glucuronide mono-conjugate
HLysPdG	hemin-lysine-pregnanediol glucuronide mono-conjugate
HMet(di)	hemin-methionine di-conjugate
HMetME	hemin-methionine methyl ester mono-conjugate
HPheME	hemin-phenylalanine methyl ester mono-conjugate
HProME	hemin-proline methyl ester mono-conjugate
HPE1G	hemin-diaminopolyethyleneglycol-estrone glucuronide mono-conjugate
HPAE1G	hemin-diaminopolyethyleneglycol-6-amino-hexanoate-estrone glucuronide mono-conjugate

HPAAE1G	hemin-diaminopolyethyleneglycol-6-amino-hexanoate-6-aminohexanoate-estrone glucuronide mono-conjugate

Arg	arginine
Asp	aspartic acid
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Leu	leucine
Lys	lysine
Phe	phenylalanine
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine