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SOLID PHASE PEPTIDE SYNTHESIS ON A BEADED CELLULOSE SUPPORT

A thesis presented in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Chemistry
at Massey University

DARREN ROSS ENGLEBRETSEN

1992

SOLID PHASE PEPTIDE SYNTHESIS ON
A BEADED CELLULOSE SUPPORT.

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ABSTRACT

The studies reported in this thesis describe the use of Perloza type MT beaded cellulose resin as an insoluble support for solid phase peptide synthesis (SPPS). The overall aim of the project was to develop a viable methodology for the synthesis of peptide-ligands directly onto Perloza for use as matrices for affinity chromatographic processes.

A number of basic studies were carried out to define the solvent compatibility of Perloza. Perloza appeared to be swollen by a variety of solvents currently used for SPPS, in particular by dimethylformamide (DMF) and dioxane. It was found that Perloza could not be dried and then re-swollen to its original volume using water, DMF, dioxane, or several other solvents. Therefore, it was necessary that Perloza was maintained in a solvent-swollen state for all of the other studies reported in this thesis.

Several methods for generation of amine-functionalised Perloza were investigated. The chosen method was reaction of Perloza with acrylonitrile in a 1:1 solution of dioxane:2% w/v NaOH to yield cyanoethyl Perloza. The level of cyanoethylation of the resin was controllable between the range of 0-3.7 mmole CN per gram of dry resin. The cyanoethyl Perloza was reduced with an excess of diborane in THF solution, either at room temperature or under reflux, to yield aminopropyl Perloza. Reduction yields varied from 52-100%.

The peptide LAGV was synthesised onto aminopropyl Perloza using modified Boc SPPS methodology. It was found that protic Boc cleavage reagents gave cleavage of aminopropyl groups from functionalised Perloza. Therefore, a novel Boc cleavage reagent, boron trifluoride etherate in dioxane, was developed for Boc cleavage in all subsequent peptide syntheses using Perloza and the Boc methodology.

C-terminal Boc-amino acids were anchored to α-bromoacetamido Perloza by nucleophilic displacement of bromine via the Boc-amino acid cesium salts. The procedure resulted in anchoring of the Boc-amino acids via an acid-stable but base-labile glycolamide linkage. Two test peptides, LAGV, and Leu-enkephalin (sequence: YGGFL), were synthesised on Perloza

using a semimanual LKB Biolynx 4175 continuous flow peptide synthesiser. The peptides were cleaved using dilute NaOH solution. The tyrosine hydroxyl of Leu-enkephalin was protected as its benzyl ether, which was cleaved by catalytic hydrogenation prior to HPLC purification. The peptides were obtained in satisfactory yield after purification by HPLC. Several unsuccessful attempts were made to synthesise the Acyl Carrier Protein 65-74 sequence (VQAAIDYING) using Perloza, the glycolamide linkage, and Boc chemistry.

The glycolamide linker was also investigated for use with Perloza and Fmoc chemistry. Leu-enkephalin was synthesised using Fmoc chemistry. The tBu ether protecting the side chain hydroxyl of the tyrosine was cleaved using 95% TFA while the peptide was left anchored to the Perloza. The peptide was then cleaved using the lithium salt of βME in THF. The cleavage yield of the peptide was low, about 32%. In addition, it was found that the Perloza was difficult to filter after the treatment with TFA, that is, its flow properties had been impaired. Leu-enkephalin with the side chain hydroxyl of the tyrosine protected as a tBu ether was obtained by cleavage of the peptide with LiβME in THF. This provided preliminary evidence that side-chain protected peptides (for further use in fragment syntheses) could be obtained using the glycolamide linker with Perloza.

The Fmoc SPPS methodology was also investigated for use with Perloza. Fmoc-amino acids were anchored to aminopropyl Perloza via the 4-hydroxymethylphenoxyacetyl (HMPA) linker using the preformed Fmoc-amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl esters. All 20 Fmoc-amino acids were anchored to Perloza at substitution levels suitable for SPPS (up to 0.76 mmole amino acid per g of dry resin). The amide linker compound p-[(R,S)-α-(9H-fluoren-9-yl)-methoxyformamido-2,4-dimethoxybenzyl]-phenoxyacetic acid was coupled to aminopropyl Perloza for syntheses of peptide amides. Both a semimanual continuous flow (LKB Biolynx 4175) and automated batchwise peptide synthesiser (ABI 430A) were used to carry out peptide syntheses. Little difference was seen in the quality of crude peptides derived from the two synthesisers. TFA solutions containing scavengers were used to cleave all peptides. It was found in all cases that treatment of peptide-Perloza with TFA seriously degraded the properties of the resin, in some cases the resin dissolved into the TFA. The peptides were purified by HPLC. Several peptides (LHRH, ACTH 4-11, Angiotensins I and II) were synthesised on Perloza and compared with authentic samples obtained from a commercial source. In addition, a number of non-standard peptides, up to 21 amino acids in length, were successfully synthesised using the Fmoc SPPS methodology with Perloza.

Two peptide-ligands were synthesised directly onto aminopropyl Perloza for testing of the peptide-Perloza conjugates as affinity matrices for biomolecule purifications. VdLPFFVdL-amidopropyl Perloza was synthesised using Boc chemistry. The peptide-Perloza was tested for binding of chymosin. It was found that chymosin would not bind to the peptide-Perloza conjugate without succinylation of the N-terminal amine group. The peptide-Perloza was used for the affinity isolation of recombinant chymosin from a solution containing a number of contaminant fungal proteins.

The side chain protected peptide luteinising hormone-releasing hormone (protected LHRH, sequence pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-NH_2) was synthesised directly onto aminopropyl Perloza. The side chain protecting groups (Trt, 2X tBu, Mtr) were cleaved quantitatively using an acidic reagent (80% DCM, 16% TFA, 1% TMSBR, 1% thioanisole, 1% EDT, 1% m-cresol), with insignificant cleavage of the peptide-ligand from the support, and no apparent impairment of the flow properties of the Perloza. The peptide-resin was then employed for the affinity isolation of antibodies to LHRH derived from a sheep immunised with LHRH conjugated to BSA.

A search of the literature revealed that, in many cases, the C-terminal glycine-amide of LHRH was required for binding antibodies to LHRH. A novel means for directed immobilisation of peptide-ligands to α-bromoacetamido Perloza was conceived and investigated in order to direct the C-terminal glycine amide into the aqueous phase. A cysteine-containing analogue of LHRH (Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was synthesised using amide linker Perloza and Fmoc chemistry. The LHRH analogue was purified by HPLC. Reaction of a 1.3-1.5X excess of the LHRH analogue with α-bromoacetamido Perloza in 0.1M $NaHCO_3$ solution resulted in anchoring of the peptide to the support via a stable thioether bond. The coupling reaction went in greater than 95% yield in 1-2 hours. The peptide-Perloza conjugate was used for the successful isolation of antibodies to LHRH.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
ABI	Applied Biosystems, Inc.
Ac	acetyl
Acm	acetamidomethyl
ACP	acyl carrier protein
ACTH	adrenocorticotropin
2AES	2-aminoethylsulphuric acid
Ag	antigen
AU	Absorbance units
AUFS	Absorbance units at full scale
Bzl	benzyl
BF_3/dioxane	boron trifluoride etherate in dioxane
βME	2-mercaptoethanol
Boc	tertiary-butyloxycarbonyl
BOP	benzotriazo-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate
BrZ	2-bromobenzyloxycarbonyl
BSA	bovine serum albumin
CDI	1,1'-carbonyldiimidazole
CE	capillary electrophoresis
CHO	formyl
ClZ	2-chlorobenzyloxycarbonyl
CMV	cytomegalovirus
DAH	1,6-diaminohexane
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
dec.	decomposed
DIC	diisopropylcarbodiimide
DIEA	diisopropylethylamine
DMA	dimethylacetamide
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMS	dimethylsulphide
Dnp	2,4-dinitrophenyl

DS	degree of substitution
EDT	ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EDC	1-ethyl-3-(dimethylaminopropyl)-carbodiimide
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2, dihydroquinoline
fac.	facing page
Fmoc	9-Fluorenylmethoxycarbonyl
FSH	follicle stimulating hormone
G	guanidino
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate
HIC	hydrophobic interaction chromatography
HMPA	4-hydroxymethylphenoxyacetic acid
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography-mass spectroscopy
IgG	immunoglobulin G
im	imidazole
in	indole
K_{acr}	$\frac{\text{volume acrylonitrile}}{\text{weight Perloza + volume dioxane + volume acrylonitrile}}$
LH	luteinising hormone
LHRH	luteinising hormone-releasing hormone
LiβME	lithium salt of 2-mercaptoethanol
M	moles per litre
MAF	Ministry of Agriculture and Fisheries
MeOH	methanol
min	minutes
MS	mass spectroscopy
MSA	methanesulphonic acid
Mtr	N^G-4-methoxy-2,3,6-trimethylbenzenesulphonyl
Mts	mesitylene-2-sulphonyl
MW	molecular weight
n.d.	not determined
NEt_3	triethylamine
NMI	N-methyl imidazole

NMM	N-methyl morpholine
OBt	benzotriazoyl ester
OBzl	benzyl ester
OtBu	tertiary-butyl ester
P	Perloza
PAM	phenylacetamidomethyl
pCl-CBZ	4-chlorocarbobenzoxy
PEG	polyethyleneglycol
Pmc	N^G-2,2,5,7,8-pentamethylchroman-6-sulphonyl
pMeO-Bzl	4-methoxybenzyl
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPPS	solid phase peptide synthesis
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate
tBu	tertiary-butyl
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulphonic acid
THF	tetrahydrofuran
Tlc	thin layer chromatography
TMSBR	trimethylsilylbromide
TNBS	trinitrobenzenesulphonic acid
Tos	4-toluenesulphonyl (= tosyl)
Trt	triphenylmethyl (= trityl)
TSGT	thermal-sol-gel-transition
UV	ultraviolet
vis	visible
WISP	Waters Intelligent Sample Programmer
Z	Benzyloxycarbonyl